

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of	)	<b>MAIL STOP</b>
Ming-Bo Wang et al.	)	<b>APPEAL BRIEF - PATENTS</b>
Application No.: 09/287,632	)	Group Art Unit: 1635
Filed: April 7, 1999	)	Examiner: JANE J ZARA
For: METHODS AND MEANS FOR	)	Appeal No.: _____
OBTAINING MODIFIED	)	
PHENOTYPES	)	
	)	

**APPEAL BRIEF**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

This appeal is from the decisions of the Examiner dated May 11, 2009 and December 8, 2009.

In the Office Action dated May 11, 2009 claims 22, 26, 42, 53, 54, 56, 58, 63-69, 85-97, 100-103, 106-110, 115-122, and 127-134 were finally rejected. A Notice of Appeal was filed November 5, 2009 together with an amendment canceling claims 70-97, 104-108, 110 and 123-134 in order to reduce the number of claims on appeal.

The final rejection of claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109, and 115-122, which are reproduced in the Claims Appendix of this brief was reiterated on December 8, 2009. This paper is timely filed April 8, 2010 in conjunction with a renewed Notice of Appeal and a petition for one months extension of time.

The Appeal Brief fee of \$540 is being paid by credit card through the e-filing payment system. The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800.

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## **I. Real Party in Interest**

Commonwealth Scientific And Industrial Research Organisation, Campbell, Australia is the real party in interest, and is the assignee of Application No. 09/287,632. Bayer CropScience AG is a licensee of the application.

## **II. Related Appeals and Interferences**

The Appellants' legal representative, or assignee, does not know of any other appeal or interferences which will affect or be directly affected by or have bearing on the Board's decision in the pending appeal. However, for the sake of completeness, Appellants note the following matters which may be of interest to the Board.

A suggestion of interference pursuant to 37 C.F.R. § 41.202 has been filed in related co-pending application 11/364,183. At the time of this appeal, the interference has not been declared.

An appeal has been filed in the re-examination of U.S. Patent No. 6,573,099, which is not directly related to the present application, but is owned by the same real party in interest. The reexamination is being conducted under application numbers 90/007,247 and 90/008,096 (merged). The appeal has been forwarded to the Board of Patent Appeals and Interferences for docketing.

## **III. Status of Claims**

Claims 1-134 have been presented in this application.

Claims 11, 13-21, 23-25, 27-39, 41, 45, 47-49, 51, 52, 55, 57, 59-62, 70-97, 104-108, 110, and 123-134 have been canceled.

The claims pending in the application are 1-10, 12, 22, 26, 40, 42-44, 46, 50, 53, 54, 56, 58, 63-69, 98-103, 109, and 111-122.

Claims 1-10, 12, 40, 43, 44, 46, 50, 98, 99, and 111-114 have been withdrawn from consideration pursuant to a restriction requirement.

Claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109 and 115-122 stand rejected and are the subject of this appeal.

#### **IV. Status of Amendments**

In the Office Action dated May 11, 2009, claims 22, 26, 42, 53, 54, 56, 58, 63-69, 85-97, 100-103, 106-110, 115-122, and 127-134 were finally rejected.

An amendment was filed November 5, 2009 canceling claims 70-97, 104-108, 110 and 123-134 to reduce the number of claims on appeal.

The amendment dated November 5, 2009 was entered by the Examiner in the Office Action dated December 8, 2009.

The final rejection of claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109 and 115-122 was reiterated by the Examiner in the Office Action dated December 8, 2009 and is the subject of this appeal.

#### **V. Summary of the Claimed Subject Matter**

A concise explanation of the subject matter defined in each of the independent claims involved in the appeal, referring to the specification by page and line number, and to the drawings, if any, by reference characters is provided below.

References to exemplary description in the specification are inserted in bold in curly brackets {} within the language of the independent claims. The references to the specification and/or drawings in the summary of the claimed subject matter is merely exemplary and should not be taken as limiting the claims in any way.

Claim 22 is directed to a plant cell, comprising a nucleic acid of interest {**p. 17, ll. 28-30**}, which is normally capable of being phenotypically expressed {**p. 16, ll. 22-26**}, further comprising a chimeric DNA molecule {**p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2**} comprising the following operably linked parts:

- a) a promoter, operative in said plant cell {**p. 15, ll. 24-29**};
- b) a DNA region {**p. 19, ll. 17-24**}, which when transcribed {**p. 15, l. 30 – p. 16, l. 6**}, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising

i) a sense nucleotide sequence {p. 20, l. 18 – p. 21, l. 11} including at least 20 consecutive nucleotides having 100% sequence identity {p. 15, ll. 9-23} with at least 20 consecutive nucleotides of the nucleotide sequence of the nucleic acid of interest; and

ii) an antisense nucleotide {p. 21, ll. 12-18} sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA {p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2} structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence,

and wherein said DNA region comprises an intron heterologous to said sense nucleotide sequence {p. 23, ll. 7-15}; and

c) a DNA region involved in transcription termination and polyadenylation {p. 29, ll. 24-30}.

Claim 63 is directed to a chimeric DNA {p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2} comprising the following operably linked parts:

a) a promoter, operative in a plant cell {p. 15, ll. 24-29};

b) a DNA region {p. 19, ll. 17-24}, which when transcribed {p. 15, l. 30 – p. 16, l. 6}, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure {p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2} comprising two annealing RNA sequences,

wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence {p. 20, l. 18 – p. 21, l. 11} identical {p. 15, ll. 9-23} to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest {p. 17, ll. 28-30},

and wherein the second of said annealing RNA sequences comprises an antisense sequence {p. 21, ll. 12-18} identical to at least 20 consecutive

nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest,

and wherein said DNA region comprises an intron heterologous to said sense sequence {p. 23, ll. 7-15}; and

c) a DNA region involved in transcription termination and polyadenylation {p. 29, ll. 24-30}.

Claim 64 is directed to a chimeric DNA {p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2} comprising the following operably linked parts:

- a) a promoter, operative in a plant cell {p. 15, ll. 24-29};
- b) a DNA region {p. 19, ll. 17-24}, which when transcribed {p. 15, l. 30 – p. 16, l. 6}, yields an RNA molecule with a nucleotide sequence comprising
  - i) a sense nucleotide sequence {p. 20, l. 18 – p. 21, l. 11} including at least 20 consecutive nucleotides having 100% sequence identity {p. 15, ll. 9-23} with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest {p. 17, ll. 28-30}; and
  - ii) an antisense nucleotide sequence {p. 21, ll. 12-18} including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure {p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2} with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,

wherein said DNA region comprises an intron heterologous to said region with sense nucleotide sequence {p. 23, ll. 7-15}; and

c) a DNA region involved in transcription termination and polyadenylation {p. 29, ll. 24-30}.

Claim 100 is directed to a plant cell, comprising a nucleic acid of interest {**p. 17, ll. 28-30**}, which is normally capable of being phenotypically expressed {**p. 16, ll. 22-26**}, further comprising a chimeric DNA molecule {**p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2**} comprising the following operably linked parts:

- a) a promoter, operative in said plant cell {**p. 15, ll. 24-29**};
- b) a DNA region {**p. 19, ll. 17-24**}, which when transcribed {**p. 15, l. 30 – p. 16, l. 6**}, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i) a sense nucleotide sequence {**p. 20, l. 18 – p. 21, l. 11**} including at least 20 consecutive nucleotides having 100% sequence identity {**p. 15, ll. 9-23**} with at least 20 consecutive nucleotides of the nucleotide sequence of the nucleic acid of interest; and
  - ii) an antisense nucleotide sequence {**p. 21, ll. 12-18**} including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure {**p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2**} with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence,

and wherein said DNA region comprises an intron {**p. 23, ll. 7-15**}; and

- c) a DNA region involved in transcription termination and polyadenylation {**p. 29, ll. 24-30**}.

Claim 102 is directed to a chimeric DNA {**p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2**} comprising the following operably linked parts:

- a) a promoter, operative in a plant cell {**p. 15, ll. 24-29**};
- b) a DNA region {**p. 19, ll. 17-24**}, which when transcribed {**p. 15, l. 30 – p. 16, l. 6**}, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure {**p. 14, l. 30 – p. 15, l. 8; p. 22, l.**

**17 – p. 23, l. 2}** comprising two annealing RNA sequences,

wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence **{p. 20, l. 18 – p. 21, l. 11}** identical **{p. 15, ll. 9-23}** to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest, and wherein the second of said annealing RNA sequences comprises an antisense sequence **{p. 21, ll. 12-18}** identical to at least 20 consecutive nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest,

and wherein said DNA region comprises an intron **{p. 23, ll. 7-15}**;  
and

c) a DNA region involved in transcription termination and polyadenylation **{p. 29, ll. 24-30}**.

Claim 103 is directed to a chimeric DNA **{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2}** comprising the following operably linked parts:

- a) a promoter, operative in a plant cell **{p. 15, ll. 24-29}**;
- b) a DNA region **{p. 19, ll. 17-24}**, which when transcribed **{p. 15, l. 30 – p. 16, l. 6}**, yields an RNA molecule with a nucleotide sequence comprising
  - i) a sense nucleotide sequence **{p. 20, l. 18 – p. 21, l. 11}** including at least 20 consecutive nucleotides having 100% sequence identity **{p. 15, ll. 9-23}** with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest; and
  - ii) an antisense nucleotide sequence **{p. 21, ll. 12-18}** including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure **{p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2}** with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive

nucleotides of the antisense sequence,  
and wherein said DNA region comprises an intron {p. 23, ll. 7-15}; and  
c) a DNA region involved in transcription termination and  
polyadenylation {p. 29, ll. 24-30}.

## **VI. Grounds of Rejection to be Reviewed on Appeal**

### **A. Written Description**

Claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109, 115-122 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. Appellants request that the rejection be overturned for the reasons set forth below.

### **B. Obviousness**

#### **1. Rejection over Fire et al., Brown et al., Lusky et al., Schiedner et al., and Baracchini et al.**

Claims 22, 26, 42, 53-54, 56, 58, 63-69, 100-103, 109, and 115-122 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Fire et al. (US 6,506,559) in view of Brown et al. (US 5,859,347), Lusky et al. (US 6,350,575) and Schiedner et al. (Nature Genetics, 18:180-83, 1998), the combination in view of Baracchini et al. (US 5,801,154). Appellants request that the rejection be overturned for the reasons set forth below.

#### **2. Rejection over Flavell, Metzlaff et al., Stam et al., Brown et al. and Lusky et al.**

Claims 22, 26, 42, 53-54, 56, 58, 63-69, 100-103, 109, and 115-122 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Flavell (Proc. Natl. Acad. Sci., 91:3490-96, 1994), Metzlaff et al. (Cell, 88:845-54, 1997) and Stam et al. (Annals of Botany, 79:3-12, 1997), the combination in view of Brown et al., (US 5,859,347), and Lusky et al. (US 6,350,575). Appellants request that the rejection be overturned for the reasons set forth below.



### **C. Double Patenting**

Claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109, and 115-122 stand provisionally rejected under the doctrine of obviousness type double patenting over pending claims 22-29, 35-38 of U.S. Patent Application No. 11/841,737. Appellants request that the rejection be overturned for the reasons set forth below.

## **VII. Argument**

For the reasons set forth below, Appellants request that the Board issue an order overturning each of the grounds of rejection in the application.

### **A. Written Description**

Claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109, and 115-122 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement for the reasons alleged in the Office Actions dated September 19, 2008, May 11, 2009, and December 8, 2009.

In the first assertion of the rejection, the Examiner made specific reference to the breadth of the limitations regarding the length and homology of the sense and antisense sequence portions and the recitation of an intron optionally located between the sense and antisense sequences. OFFICE ACTION dated November 10, 2005, at 7. The Examiner concluded that:

The specification fails to teach or adequately describe a representative number of species such that the common attributes or characteristics concisely identifying members of the proposed genus are exemplified. And because the claimed genus is so highly variant, the description provided is insufficient.

*Id.* The Examiner acknowledged that the specification teaches fully complementary pair constructs for reducing the phenotypic expression of a transgenic GUS gene and for reducing the phenotypic expression of the  $\Delta 12$  desaturase target gene in Arabidopsis which complementary pair constructs additionally comprise the pyruvate orthophosphate dikinase 2 intron from *Flaveria trinervia* in forward or reverse orientation. Office Action Mailed February 8, 2006, at 4, lines 10-22. However, the Examiner has maintained that the specification fails to teach or adequately describe a representative number of species in the

genus such that the common attributes or characteristics concisely identifying members of the proposed genes are exemplified. *Id.*

The Examiner's conclusion appears to rest on an improper interpretation of the written description requirement as *per se* requiring that some greater number of representative specific formulae are exemplified in order to adequately describe any generic invention comprising chemical constructs.

Appellants respectfully submit that the Examiner has not appropriately considered the fundamentally general nature of the invention or how the invention would be understood from the standpoint of one skilled in the art. Satisfaction of the written description requirement must be determined from the standpoint of one of skill in the art at the time the application was filed. *See, e.g., Wang Labs. v. Toshiba Corp.*, 993 F.2d 858, 865, 26 U.S.P.Q.2d 1767, 1774 (Fed. Cir. 1993). When the fundamental nature of the invention as a generalized method of constructing tools for the control of gene expression from widely known and freely available parts is considered from the standpoint of one skilled in the art, the specification is more than sufficient to demonstrate that the inventors were in possession of the genus of tools claimed in the form of nucleic acid constructs and cells comprising those constructs.

Showing possession of the claimed invention means describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas so as to fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 U.S.P.Q.2d 1961, 1966 (Fed. Cir. 1997). Generally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement. Information which is well known in the art need not be described in detail in the specification. *See, e.g., Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986).

Appellants respectfully submit that the specification sets forth a description of the structure and function of the claimed nucleic acid constructs and cells and how to make them that a person of ordinary skill in the art would immediately recognize the constructs with all their limitations and know how to make and use the constructs and cells. The constructs themselves can be assembled from a wide variety of freely available and well known nucleic acid sequence components. It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided

that the effect is sufficiently demonstrated to characterize a generic invention. *Capon v. Eshhar*, 76 U.S.P.Q.2d 1078, 1085 (Fed. Cir. 2005)(citing *In re Angstadt*, 537 F.2d 498, 504, 190 U.S.P.Q. 214 (C.C.P.A. 1976)).

The Examiner has recognized that the effect of the invention has been demonstrated by examples in the specification. OFFICE ACTION dated November 10, 2005, at 7. The Examiner has recognized that the claimed constructs are not encoding enzymes whose function could be abolished or at least compromised by slight or subtle sequence changes. OFFICE ACTION dated June 22, 2006, at 4. Combining the teaching of the specification with the knowledge available in the art of many potential target genes, a person of ordinary skill would have appreciated that the invention encompassed any species of the claimed genus of chimeric constructs that have the recited structural features. It would not have been practical nor was it necessary for Appellants to list every known sequence that could be used in making a construct according to the claimed invention. *See Capon v. Eshhar*, 76 U.S.P.Q.2d 1078, 1085-86 (Fed. Cir. 2005). Appellant's disclosure provided not only general teachings of how to select and recombine DNA, but also specific examples of the production and use of the claimed chimeric genes. According to the precedent of this Board's reviewing Court, that is a written description sufficient to support the generic claims. *Id.*

The specification provides adequate written description support for every individual aspect of the claimed invention and the claims taken as a whole. Consider, for example, claim 64, which recites:

A chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in a plant cell;
  - b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising
    - i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest; and
    - ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;
- wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive

nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,  
wherein said DNA region comprises an intron heterologous to said region with sense nucleotide sequence;  
and  
c) a DNA region involved in transcription termination and polyadenylation.

Each element and limitation of the claim is described in the specification. Chimeric DNA are generally described, for example at p. 11, ll. 4-20, p. 18, ll. 7-16 and p. 26, l. 28 to p. 27, l. 2. The examples of the application illustrate specific embodiments of such chimeric DNA constructs. There is no evidence that that a person of ordinary skill in the art would not have recognized that the inventors were in possession of such chimeric DNA and cells comprising the chimeric genes.

The various parts comprising the chimeric DNA are described throughout the specification with all the limitations set forth in the claims. The chimeric DNA is comprised of the following parts:

A promoter, operative in a plant cell, as described for example at p. 15, ll. 24-29. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors were in possession of a wide variety of suitable promoters.

A DNA region, which when transcribed, yields an RNA molecule as described for example at p. 15, l. 30 to p. 16, l. 6, and p. 19, ll. 17-24 and shown in the examples. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors had described DNA regions, which when transcribed would yield a desired RNA molecule.

A sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest as described for example at p. 15, ll. 9-23, p. 17, ll. 28-30, and p. 20, l. 18 to p. 21, l. 11 and shown in the examples. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors had described sense sequences of at least 20 nucleotides that were identical to a nucleic acid of interest. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that a broad genus of nucleic acids of interest such as described in the specification was known at the time the application was filed.

An antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence as described, for example at p. 21, ll. 12-18 and shown in the examples. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors had described antisense sequences corresponding to the recited sense sequences and including all the limitations of the claims.

An intron heterologous to the sense nucleotide sequence as described for example at p. 23, ll. 7-15 and shown in the examples. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors had described the chimeric constructs as comprising an intron that was heterologous to a nucleic acid of interest. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that a broad genus of introns was known at the time the application was filed.

A DNA region involved in transcription termination and polyadenylation as described, for example, at p. 29, ll. 24-30 and shown in the examples. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors had described chimeric constructs comprising a DNA region involved in transcription termination and polyadenylation. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that a broad genus of DNA regions involved in transcription termination and polyadenylation was known at the time the application was filed.

The claim further provides that the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence. The provision for such artificial hairpin structures in an RNA transcribed from a chimeric DNA is described in the specification, for example, at p. 14, l. 30 to p. 15, l. 8, and p. 22, l. 17 to p. 23, l. 2. The Examiner has provided no evidence that a person of ordinary skill in the art would not have recognized that the inventors had described chimeric constructs in which the resulting RNA would form an artificial hairpin.

Thus, each and every element of the invention as set forth in the claim is described in the specification. The individual components are constructed of widely available materials that were known to those of skill in the art. The combination and arrangement of the

elements is described in the specification such that a person of ordinary skill in the art would immediately recognize the claimed constructs as what the inventors invented. A person of ordinary skill in the art would have recognized that the description of the recited structural features of the claimed DNA could be combine with the knowledge in the art of any of the myriad known gene sequences to provide a useful chimeric DNA.

Such a description satisfies the principle of the written description requirement as explained by the Federal Circuit. *See Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 U.S.P.Q.2d 1078, 1084 (Fed. Cir. 2005) (“The ‘written description’ requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor’s obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed.”)

When the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh. *Capon v. Eshhar*, 418 F.3d at 1358. Rather, the Court explained that:

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

*Id.* at 1357. Indeed, the Federal Circuit has recognized that a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. *Falkner v. Inglis*, 79 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2006)(citing *Capon*, *supra*). Because, each of the elements of the claims is described in the specification, and each of the elements may be constructed from widely known and available materials, there is no need for description of additional representative species.

## **1. Evidence in the Record Proves the Sufficiency of the Disclosure**

### **(a) Generalized Use By Those of Skill in the Art Based Upon the Disclosed Examples**

A selection of publications reporting chimeric DNA constructs comprising artificial hairpin sequences made to silence a variety of nucleotide sequences of interest and using

various intron sequences in accordance with the disclosure of the present invention have been submitted for the record. *See* Exhibits 10-18. Most of these publications make explicit reference to Smith et al. 2000, *Nature*, 407, 319-320 (EXHIBIT 9). This publication, which was co-authored by named inventors of the application, presented the scientific publication of the examples disclosed in the current application. The submitted publications represent a sample of publications that give due credit to the scientific publication of the examples disclosed in the present specification as providing disclosure leading to widespread general manufacture and use of the claimed constructs.

The generalized nature of the invention described in the specification is evidenced by the widespread scientific publication of numerous reports describing the making and use of constructs of the kind described in specification using a variety of materials known to those of skill in the art. Thus, these publications evidence the recognition of persons of skill in the art that the chimeric DNA constructs of the invention can be made using any known intron sequence has been borne out by subsequent publication. For example, Wesley et al. 2001, *The Plant Journal*, 27:581-90(EXHIBIT 10) demonstrates that those skilled in the art recognized that an intron sequence other than the pyruvate orthophosphate dikinase 2 intron 2 exemplified in the specification can be used. The table on page 587 of the publication indicates efficient silencing using at least two other introns ( $\Delta$ 12a and  $\Delta$ 12c introns).

This publication further demonstrates that persons skilled in the art recognized that the location of the intron is not critical. Indeed, as pointed out on page 585 of this publication, sentence spanning the 1<sup>st</sup> and 2<sup>nd</sup> column, the presence of an intron in the 5' portion of the transcribed region, in the absence of any further intron between the sense and antisense strands, led to efficient silencing.

Samuel and Ellis 2002, *The Plant Cell*, 14: 2059-69 (EXHIBIT 11) describe efficient gene silencing in plants using a hairpin construct as described by Smith et al. 2000 (see page 2064 1st column, three lines from bottom), and as described in the present specification, comprising the fourth intron of AtMPK6 gene (page 2066, 2nd column, section entitled "intron spliced Hairpin loop RNA-SIPK construct", lines 3-5).

Acosta-Garcia and Vielle-Calzada 2004, *The Plant Cell*, 16: 2614-28 (EXHIBIT 12) describe efficient gene silencing in plants using a hairpin construct as described by Smith et al. 2000 (page 2617, 2nd column, line 17), and as described in the present specification, comprising an intron of chalcone synthase gene (page 2617, 2nd column, lines 13-14, page 2625, 2nd column, lines 20-22 and Fig 4A - page 2619).

Guo et al., 2003, *The Plant Journal*, 34: 383-92 (EXHIBIT 13) describe efficient gene silencing in plants using a hairpin construct as described by Smith et al. 2000 (page 383, sentence spanning 1st and 2nd column), and as described in the present specification, comprising the third intron of the actin 11 gene (Figure 1, page 384; legend to Figure 1, page 384, line 8 and page 391, 1st column, section entitled "Plasmid construction", lines 2-4).

Chen et al., 2003, *The Plant Journal*, 36: 731-40 (EXHIBIT 14) describe efficient gene silencing in plants using a hairpin construct as described by Smith et al. 2000 (page 732, 1st column, line 33), and as described in the present specification, comprising intron 1 of potato GA20 oxidase gene (Figure 1, page 733 legend to figure 1; page 733, lines 6-7 and page 738 1st column section entitled "Plasmid construction and plant transformation", lines 8-9).

Byzova et al., 2004, *Planta*, 218:379-87 (EXHIBIT 15) describe efficient gene silencing in plants using a hairpin construct as described by Smith et al. 2000 (page 384, 2nd column, line 9), and as described in the present specification, comprising Intron IV2 from the potato ST-LS1 gene (page 380, 2nd column, section entitled "Plasmid construction", lines 18-19).

Lee et al., 2003, *Methods*, 30: 322-29 (EXHIBIT 16) describe efficient gene silencing in animals using a hairpin construct as described by Smith et al. 2000 (page 324, 2nd column, lines 1-3), and as described in the present specification, comprising intron 2 of the white gene (Legend to figure 4, page 327, line 1; page 324, 2nd column, lines 31-34)

Further examples of efficient gene silencing using hairpin constructs with introns are provided by Li et al., 2005, *The Plant Cell*, 17:859-75 (EXHIBIT 17) (using the first intron of the GhTUB1 gene-page 873 1st column, 4th section) and O'Brien et al. 2002, *The Plant Journal*, 32:985-96 (EXHIBIT 18) (using an intron from HD2 histone deacetylase gene-page 994, 2nd column lines 9-10).

This selection of publications reporting work using hpRNA constructs comprising introns as taught by the present application and reported by Smith et al. 2000 prove that any known nucleic acid sequence of interest can be silenced using hpRNA constructs comprising any intron in accordance with the written description and claims.

These widespread publications by a variety of scientists demonstrate that combining the teaching of the specification with knowledge available in the art, a person of ordinary skill would have appreciated that the inventors had invented the claimed genus of chimeric constructs having the recited structural features and further comprising any intron in any position. Appellants' application described the essential structure and function of a new molecular tool and taught those of skill in the art how to select and recombine known DNA



structures to make and use their invention. Appellants also provided illustrative working examples of the claimed chimeric genes. The sampling of subsequent publications in the art presented by Appellants demonstrated that the general applicability of the invention has been borne out over time.

**(b) Affidavits Presented To The Examiner Prove The Sufficiency Of The Disclosure**

Appellants also submitted the declarations of Dr. Elizabeth Salisbury Dennis (EXHIBIT 19) and Dr. Marc de Block (EXHIBIT 20), experts in the field, presented pursuant to 37 C.F.R. § 1.132 which further prove that the teachings of the specification would have demonstrated to a person of ordinary skill in the art that the elements of the invention recited in the claims are sufficient for the claimed chimeric DNA to perform its intended function over the full range encompassed by the claims, i.e. to permit suppression of the expression of any gene of interest using any intron sequence anywhere in the transcribed region of the dsRNA encoding chimeric gene. These experts have testified that the teachings of the specification would have permitted a person of ordinary skill in the art to make and use a range of chimeric DNA constructs consistent with the scope of the claims from components that were known in the art including any of a large number of well known introns.

For example, with regard to the potential breadth of the genus of intron sequences comprised in the constructs, as noted by Dr. Dennis, the Application teaches at least on page 23, lines 5 to 15 that the chimeric DNA constructs of the invention may comprise an intron in the transcribed region that encodes the double-stranded RNA molecule, that the inclusion of the intron enhances the efficiency of reduction of expression of the target nucleic acid interest, and that the intron is preferably (but not necessarily) located in the spacer region. The Application also teaches on page 23, lines 13 to 15 that the intron in a “particularly preferred embodiment” is the *Flaveria trinervia* pyruvate orthophosphate dikinase 2 intron 2 as used in Example 6. Declaration of Dr. Elizabeth Salisbury Dennis at ¶ 11.

Thus, Dr. Dennis and Dr. de Block have testified that a person of ordinary skill in the art would have recognized that the description of the recited structural features of the claimed DNA could be combined without limitation with the knowledge of any of the myriad of sequences encompassed by the genus intron and further with the knowledge that insertion of such intron sequence anywhere in the transcribed region of the claimed gene would result in identical dsRNA molecules. Declaration of Dr. Elizabeth Salisbury Dennis at ¶ 16.

Dr Dennis is an expert on the use of introns in genetic constructs. DECLARATION OF DR. ELIZABETH SALISBURY DENNIS at ¶¶ 1-3 and Exhibit 1 attached thereto. Dr. Dennis elaborates on the following points:

- That numerous introns from a wide range of eukaryotic organisms and genes were known and characterized at the date of filing the application. *Id.* at ¶¶ 12, 14
- That introns were known to be removed from primary transcripts by a universally conserved RNA splicing pathway. *Id.* at ¶13.
- That the important structural features for the removal of introns from primary transcripts were known to be highly conserved between different introns. *Id.* at ¶13.
- That a person of ordinary skill in the art would have appreciated the interchangeability of introns in the chimeric constructs of the invention described and claimed in the application. *Id.* at ¶14.
- That it would have been appreciated at the time of the application that intron sequences are removed from the primary transcripts during splicing and that the resultant spliced dsRNA molecule would be the same irrespective of the exact position of the intron in the transcribed region. *Id.* at ¶15.

Dr. Dennis testified that because of the conserved nature of intron processing, a person of ordinary skill in the art would have immediately appreciated the general interchangeability of the intron that is taught in the specification, i.e. that the intron specifically exemplified in the Application is exchangeable with any other intron sequence. *Id.* at ¶14. Furthermore, Dr. Dennis concluded that a person of ordinary skill in the art would have appreciated and expected that the chimeric genes encoding dsRNA molecules would be functional irrespective of the position of the intron in the transcribed region. *Id.* at ¶15. Accordingly, it is her opinion that when the person of ordinary skill in the art read the teachings of the present application, which include the general usefulness of including an intron in the chimeric DNA, it would have been immediately clear to such person that the teaching of the Application is not limited to the specifically exemplified intron sequence, nor the exemplified preferred location for the intron. *Id.* at ¶16.

Dr Dennis further testified that her opinion is supported by numerous publications, such as those provided by Appellants with the Amendment and Reply filed November 22,

2006 and included as attachments to her declaration, that followed the results first disclosed in the present application. Dr. Dennis noted that several publications reported that by following teachings of the application that were also reported in Smith et al. (2000), persons of ordinary skill in the art were successful in using a variety of intron sequences in the transcribed region of chimeric DNA such as presently claimed. *Id.* at ¶17 and Exhibits 3-12 attached thereto.

Dr Marc De Block declared that that in his opinion, based upon personal experience, that a person skilled in the art would have immediately understood that the applicability of the inventions described in the Application is not limited to the specific Example therein, nor to the particular intron used in that Example. DECLARATION OF DR. MARC DE BLOCK at ¶17. Dr. De Block testifies that a person of ordinary skill in the art would have understood that the exemplified intron could be exchanged for other well known introns (which would function in the same manner, since the process of removal of introns from RNA molecules is a conserved process). *Id.* at ¶14. His opinion is supported by his personal experience upon learning of the results disclosed in the present application and applying that learning to his own project in the context of a licensing of the invention described in the application and subsequent collaboration with the inventors. *Id.* ¶ 10-12.

Furthermore, his own reaction to the disclosure of the invention by the inventors is evidence of the broadly applicable nature of the inventor's disclosed discoveries. Indeed, upon being informed of the increased efficiency in gene-silencing obtained by the inclusion of an intron in the transcribed region of the dsRNA encoding chimeric gene Dr De Block immediately included an intron in the design of the gene silencing constructs for the particular project he was working on. *Id.* at 12. Dr. De Block did not include the exemplified pyruvate orthophosphate dikinase 2 intron from *Flaveria trinervia*, but instead used another well known intron sequence with similar effect. *Id.* at ¶¶13-14.

In further response to arguments made by the Examiner, an additional declaration by Dr Peter Schofield (EXHIBIT 21) was submitted further supporting Appellants' position that the specification includes a written description of the claimed invention such that a person of ordinary skill in the art would have recognized that the inventors were in possession of the claimed invention at the time that the application was filed. Dr. Schofield's declaration provides further clear evidence that the term "intron" defined a well characterized genus of DNA elements possessing common structural and functional features.

The genus of intron included a large and representative number of well characterized members having well understood common structural and functional properties. A person skilled in the art clearly knows and would have known that what is defined by an “intron” includes the presence of consensus sequences at both ends of the intron for intron splicing. *See, e.g.,* DECLARATION OF ELISABETH DENNIS at ¶ 13; DECLARATION OF PETER SCHOFIELD at ¶¶ 13 and 15.

These declarations further prove that the teachings of the present application satisfy the written description requirement of 35 U.S.C. § 112, first paragraph as explained in *Capon v. Eshhar*, “[t]he ‘written description’ requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed.” *Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 U.S.P.Q.2d 1078 (Fed. Cir. 2005). Both Dr. Dennis and Dr. De Block have testified and provided reasons as to why the present application satisfies the requirement.

Appellants respectfully submit that each declaration presented herewith effectively refutes the allegation by the Examiner that the specification does not adequately describe chimeric DNA comprising any intron inserted anywhere in the transcribed region of the claimed chimeric DNA that would perform the functions asserted in the specification. The testimony of both Dr. Dennis and Dr. De Block evidences the fact that the disclosure of the present specification would have been sufficient to describe the necessary and distinguishing features of the claimed invention to a skilled person. This too, is evidenced by testimony in the record. In response to yet further arguments by the examiner, Appellants submitted the declaration of Dr Michael Metzloff (EXHIBIT 22), stating his opinion that the application described the invention in sufficient detail to demonstrate that the inventors had a complete conception of the invention and described it sufficiently such that a person of ordinary skill in the art would have understood what the invention was and how it was distinguished over the prior art. DECLARATION OF METZLAFF page 4, paragraph 14.

Dr. Metzloff was a person working in the filed at the time of the invention and adds yet another voice to the chorus of experts that testify a person of ordinary skill in the art would have recognized from the application as filed that the inventors were in possession of the invention that is currently claimed. That is all that is required when a variety of suitable component sequences are known and would be recognized by those skilled in the art. *See*

*Falkner v. Inglis*, 79 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2006)(“it is binding precedent of this court that Eli Lilly does not set forth a per se rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art”); see also *Monsanto Co. v. Scruggs*, 79 U.S.P.Q.2d 1813, 1818 (Fed. Cir. 2006)(“Given the knowledge in the art, it was unnecessary for the ‘605 patent to include specific gene sequences when referring to the CaMV 35S promoter to meet the written description requirement.”).

Thus, Appellants have presented extensive testimony from experts in the art proving that persons of skill in the art would have recognized that the descriptions and examples of the specification demonstrated that the inventors were in possession of a generic invention encompassing a novel set of tools for the manipulation of gene expression. The testimony of these experts is evidence that such tools could be constructed as described in the application and claimed in the claims for a wide range of genes of interest using known components. The Examiner has persistently failed to give adequate consideration and weight to the testimony of these experts, relying instead on mere conclusory statements that the claimed genus is too varied to be adequately described.

There was no need in the present application to describe introns other than those presented in the working examples, because at the time of filing the current application, other intron sequences were well known. The working examples of an application need not explicitly span the full scope the claim language to support the adequacy of a written description. *Falkner*, 79 U.S.P.Q.2d at 1007 (“A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before. Placed in that context, it is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation.”)(citing *Lizard Tech, Inc v. Earth Resource Mapping PTY, Inc*, 424 F.3d 1336, 1345, 76 U.S.P.Q.2d 1724 (Fed. Cir. 2005); see also *Union Oil Co. v. Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 U.S.P.Q.2d 1227 (Fed. Cir. 2000); *In re GPAC Inc.*, 57 F.3d 1573, 1579, 35 U.S.P.Q.2d 1116 (Fed. Cir. 1995)).

## 2. Each of the Independent Claims is Supported by the Specification

Exemplary support for each of the independent claims is further demonstrated by the following tables in which exemplary support for each element is listed.

### (a) Claim 22

Claim 22	Exemplary Disclosure
A plant cell, comprising a nucleic acid of interest,	{p. 17, ll. 28-30; examples}
which is normally capable of being phenotypically expressed,	{p. 16, ll. 22-26; examples}
further comprising a chimeric DNA molecule comprising the following operably linked parts	{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2; examples }
a) a promoter, operative in said plant cell;	{p. 15, ll. 24-29; examples}
b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising	{p. 19, ll. 17-24; examples} {p. 15, l. 30 – p. 16, l. 6; examples}
i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of the nucleic acid of interest; and	{p. 20, l. 18 – p. 21, l. 11; examples} {p. 15, ll. 9-23; examples}
ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;	{p. 21, ll. 12-18; examples}
wherein the RNA is capable of forming an artificial hairpin RNA structure	{p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2; examples}

with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence,	
and wherein said DNA region comprises an intron heterologous to said sense nucleotide sequence; and	{p. 23, ll. 7-15; examples}
c) a DNA region involved in transcription termination and polyadenylation.	{p. 29, ll. 24-30; examples}

**(b) Claim 63**

Claim 63	Exemplary Disclosure
A chimeric DNA comprising the following operably linked parts:	{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2; examples}
a) a promoter, operative in a plant cell;	{p. 15, ll. 24-29; examples}
b) a DNA region, which when transcribed, yields	{p. 19, ll. 17-24; examples} {p. 15, l. 30 – p. 16, l. 6; examples}
an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences,	{p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2; examples}
wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest,	{p. 20, l. 18 – p. 21, l. 11; examples}  {p. 15, ll. 9-23; examples}  {p. 17, ll. 28-30; examples}
and wherein the second of said annealing RNA sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of	{p. 21, ll. 12-18; examples}

at least part of said nucleotide sequence of said nucleic acid of interest,	
and wherein said DNA region comprises an intron heterologous to said sense sequence; and	{p. 23, ll. 7-15; examples}
c) a DNA region involved in transcription termination and polyadenylation.	{p. 29, ll. 24-30; examples}

**(c) Claim 64**

Claim 64	Exemplary Support
A chimeric DNA comprising the following operably linked parts:	{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2; examples}
a) a promoter, operative in a plant cell;	{p. 15, ll. 24-29; examples}
b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising	{p. 19, ll. 17-24; examples}  {p. 15, l. 30 – p. 16, l. 6; examples}
i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest; and	{p. 20, l. 18 – p. 21, l. 11; examples}  {p. 15, ll. 9-23; examples}  {p. 17, ll. 28-30; examples}
ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;	{p. 21, ll. 12-18; examples}
wherein the RNA is capable of forming an artificial hairpin RNA structure	{p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2; examples}



with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,	
wherein said DNA region comprises an intron heterologous to said region with sense nucleotide sequence; and	{p. 23, ll. 7-15; examples}
c) a DNA region involved in transcription termination and polyadenylation.	{p. 29, ll. 24-30; examples}

**(d) Claim 100**

A plant cell, comprising a nucleic acid of interest, which is normally capable of being phenotypically expressed, further comprising	{p. 17, ll. 28-30; examples}
a chimeric DNA molecule comprising the following operably linked parts:	{p. 16, ll. 22-26; examples}
a) a promoter, operative in said plant cell;	{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2; examples}
b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising	{p. 15, ll. 24-29; examples}
i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of the nucleic acid of interest; and	{p. 19, ll. 17-24; examples}
ii) an antisense nucleotide	{p. 15, l. 30 – p. 16, l. 6; examples}
	{p. 20, l. 18 – p. 21, l. 11; examples}
	{p. 15, ll. 9-23; examples}
	{p. 21, ll. 12-18; examples}

sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;	
wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence,	{p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2; examples}
and wherein said DNA region comprises an intron; and	{p. 23, ll. 7-15; examples}
c) a DNA region involved in transcription termination and polyadenylation.	{p. 29, ll. 24-30; examples}

**(e) Claim 102**

A chimeric DNA comprising the following operably linked parts:	{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2; examples}
a) a promoter, operative in a plant cell;	{p. 15, ll. 24-29; examples}
b) a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences,	{p. 19, ll. 17-24; examples}  {p. 15, l. 30 – p. 16, l. 6; examples}  {p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2; examples}
wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest, and	{p. 20, l. 18 – p. 21, l. 11; examples}  {p. 15, ll. 9-23; examples}
wherein the second of said annealing RNA	{p. 21, ll. 12-18; examples}

sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest,	
and wherein said DNA region comprises an intron; and	{p. 23, ll. 7-15; examples}
c) a DNA region involved in transcription termination and polyadenylation.	{p. 29, ll. 24-30; examples}

**(f) Claim 103**

A chimeric DNA comprising the following operably linked parts:	{p. 18, ll. 7-16; p. 26, l. 28 – p. 27, l. 2; examples}
a) a promoter, operative in a plant cell;	{p. 15, ll. 24-29; examples}
b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising	{p. 19, ll. 17-24; examples}  {p. 15, l. 30 – p. 16, l. 6; examples}
i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest; and	{p. 20, l. 18 – p. 21, l. 11; examples}  {p. 15, ll. 9-23; examples}
ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;	{p. 21, ll. 12-18; examples}
wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between	{p. 14, l. 30 – p. 15, l. 8; p. 22, l. 17 – p. 23, l. 2; examples}

the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,	
and wherein said DNA region comprises an intron; and	{p. 23, ll. 7-15; examples}
c) a DNA region involved in transcription termination and polyadenylation.	{p. 29, ll. 24-30; examples}

### **3. The Written Description Rejection Must Be Overturned**

Appellants have shown how the specification demonstrates that the inventors were in possession of the claimed invention at the time the application was filed by describing the invention with all its limitations in a manner that would have been recognized by persons of ordinary skill in the art.

Appellants have shown that support for the claimed invention with all of its limitations is provided in the specification.

Appellants have provided a series of scientific publications which bear out the general applicability of the invention disclosed in the specification.

Appellants have presented the testimony of experts in the art proving that persons of ordinary skill would have recognized the sufficiency and general applicability of the disclosure of the invention, and thus that the inventors were in possession of the full scope of the claimed invention.

For all these reasons the sufficiency of the description of the claimed invention is proven. Accordingly, the rejection of the claims under 35 U.S.C. § 112, first paragraph should be overturned and such action is respectfully requested.

## **B. Obviousness**

In *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1 (1966), the Court set out a framework for applying the statutory language of §103. *See* 383 U.S., at 15–17. The analysis is objective:

Under §103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented.

*Id.*, at 17–18.

In setting forth the rejections under 35 U.S.C. § 103, the Examiner has failed to accurately determine the scope and content of the prior art. In the first rejection, the Examiner has applied a reference (Fire et al.) which appellants have antedated. The Examiner's grounds for ignoring the antedating affidavit are not supported by authority, but rather are contrary to controlling precedent. In the second rejection, the Examiner has interpreted the scope and content of the prior art in a manner that is contradicted by objective evidence of how the phenomenon underlying the invention was viewed by those of ordinary skill in the art at the time the invention was made and subsequently. The Examiner's interpretation is further contradicted by the testimony of an expert in the art at the time the invention was made, a co-author of the cited art. Because the phenomenon underlying the invention would not have been, could not have been, and was not predicted at the time the invention was made, the claimed invention which represents further surprising improvements over that fundamental discovery could not possibly have been obvious.

Because the Examiner has misapprehended the fundamental determination of the scope and content of the prior art with respect to the primary references upon which an analysis under 35 U.S.C. § 103 must be based, the rejections cannot be sustained.

Furthermore, each of the claims requires inclusion of an intron in the chimeric gene construct, which the inventors have discovered provides a surprising increase in the effectiveness of the construct. Such a feature is not suggested in the primary references relied upon by the Examiner. The Examiner has cited a number of secondary references for the intron feature. However none of those references provides a reason for including an intron

that is relevant to the present invention. Rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness. *See In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006). Therefore, even if the Examiner were correct with respect to applicability of the primary references, in the absence of some cognizable reason to include an intron in the claimed chimeric gene constructs and cells, the present invention is still non-obvious, and the rejections cannot be sustained.

**1. Rejection over Fire et al., Brown et al., Lusky et al., Schiedner et al., and Baracchini et al.**

Claims 22, 26, 42, 53-54, 56, 58, 63-69, 100-103, 109, and 115-122 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Fire et al. (US 6,506,559)(EXHIBIT 1) in view of Brown et al. (US 5,859,347) (EXHIBIT 4), Lusky et al. (US 6,350,575) (EXHIBIT 5) and Schiedner et al. (Nature Genetics, 18:180-83, 1998) (EXHIBIT 7), the combination in view of Baracchini et al. (US 5,801,154) (EXHIBIT 8). In the first assertion of this rejection, the Examiner alleged that:

Fire et al. (U.S. Patent No. 6,505,559) [teaches] plant cells, plants and their seeds comprising a first and second DNA sequence which expresses in the plant cell a chimeric DNA comprising a promoter, operatively linked to a DNA region, which when transcribed yields [an] RNA molecule capable of forming a hairpin comprising two annealing RNA sequences which comprise a sense sequence sharing homology with consecutive nucleotides of a target nucleic acid of interest in the plant, and which further comprises a second, annealing RNA sequence comprising antisense sharing homology with the consecutive nucleotides of the sense strand that targets the nucleic acid of interest, and which chimeric DNA further comprises an intron sequence, and which chimeric DNA further comprises operably linked transcription termination and polyadenylation sequences (See the abstract, col. 3-4, col. 5, line 47-col. 6, line 54, col. 7, line 42-col. 9, lines 25, col. 11, line 37-col. 12, line 8, col. 17, line 20-24, col. 21, line 36-col. 22, line 4, claims 1-4 and 7).)

OFFICE ACTION dated April 11, 2005, at 4. The Examiner acknowledged that Fire et al. do not teach an intron interposed between sense and antisense strand, or the limitations of the length and identity of targeting region to the target sequence then appearing in the claims. The Examiner alleged that:

Brown et al. (U.S. Patent No. 5,859,347) teach plant cells transformed with chimeric nucleic acid constructs expressing desired DNA sequences, and which expression constructs comprising expression elements including

operably linked promoters and further comprising heterologous introns, which introns enhance expression of the desired nucleic acid sequences in the expression construct (see col. 8, line 53-col. 9, line 17, examples 1-7 in cols. 10-18 and figures 8-27).

OFFICE ACTION dated April 11, 2005, at 5. The Examiner further alleged that:

Lusky et al. (6,350,575) teach expression constructs comprising antisense RNA and further comprising an intron as well as other expression elements including translation termination and polyadenylation signals (col. 6, line 15-col. 7, line 14).

OFFICE ACTION dated April 11, 2005, at 5. The Examiner further alleged that:

Barachini et al. teach the ability to target a gene of interest with a complementary sequence comprising at least 10 nucleobases (see e.g. claims 1, 12, 26, and 32).

OFFICE ACTION dated April 11, 2005, at 5.

**(a) Fire et al. is Not Prior Art**

Appellants maintain as set forth below that Fire et al. alone, or in combination with Brown et al., Lusky et al., Schiedner et al., and Baracchini et al., would not have rendered the presently claimed invention obvious. Nevertheless, in order to remove Fire et al. as an issue, Appellants submitted a Declaration by the Inventors under 37 C.F.R. § 1.131 (EXHIBIT 23) with the Amendment filed May 1, 2008.. The declaration presents evidence that the invention was actually reduced to practice, prior to the December 23, 1997 filing date of U.S. provisional application No. 60/068,562 for which benefit is claimed by Fire et al. (U.S. Patent 6, 506, 559).

In particular, the declaration presented evidence that prior to December 23, 1997 they successfully constructed, in the CSIRO Plant Industry laboratories in Canberra, Australia, a chimeric DNA construct molecule comprising, in order: a) a promoter (*CaMV35S*), operative in a plant cell; b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising i) a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the nucleotide sequence of the nucleic acid of interest (*0.75 kb PVY region in sense orientation- the nucleic acid of interest was comprised in the genome of an infecting RNA virus in this embodiment*); and ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence

identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*); wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence, and wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and c) a DNA region involved in transcription termination and polyadenylation (*3' ocs region*). See EXHIBIT 23 at ¶¶ 15-17.

The declaration also presented evidence that prior to December 23, 1997 the inventors successfully constructed, in the CSIRO Plant Industry laboratories in Canberra, Australia, a chimeric DNA molecule (*pMBW233/239 series*) comprising in order, a promoter operative in the plant cell (*Ubi-P*); a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (*Gusd in sense orientation*) in a eukaryotic cell; and an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*Gus5' in antisense orientation*); wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (*Ubi-1 intron*) (which is heterologous to the sense GUS sequence); and a DNA region involved in transcription termination and polyadenylation (*tml'*). See EXHIBIT 23 at ¶ 38.

The declaration also presented evidence of the successful use of such a chimeric gene in a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (GUS gene) in a plant cell (*rice*) comprising the step of introducing into the plant cell, a chimeric DNA (*pMBW233/239 series*) as claimed. See EXHIBIT 23 at ¶ 39.

The Examiner has contended that the actual reduction to practice demonstrated in the Declaration of the Inventors is not effective to antedate Fire et al. because the construct made by the inventors had longer sense and antisense segments than the “at least 20 nucleotides”



recited in the claims. OFFICE ACTION dated December 89, 2009, at 12-13. The Examiner has cited no authority for this position. By contrast, in *In re Clarke*, the Court explained:

If the question of how much need be shown in antedating affidavits is cast in terms of *support* for the *claims*, then an applicant would be required to show as much as is required by 35 U.S.C. 112 to *support* a generic *claim*. However, we think such analysis in terms of support for the claims to be erroneous and improper here. We would add that an analysis in terms of the law involving “genus” and “species” does not promote a disposition of the issue here.

*In re Clarke*, 148 U.S.P.Q. 665, 669-70 (C.C.P.A. 1966). Thus, it is old law that antedating affidavits need not show the full scope of the claims, but rather “antedating affidavits must contain facts showing a completion of ‘the invention’ commensurate with the extent the invention is shown in the reference.” *Id.*

Contrary to established law, the Examiner would apply a genus / species analysis of support under 35 U.S.C. § 112 to the affidavits where controlling precedent has clearly stated that such analysis is not applicable. As acknowledged by the Examiner, Fire et al. does not show the whole invention now claimed. By contrast, the declaration of the inventors demonstrates reduction to practice of complete embodiments of the now claimed invention. Thus, it is clear that the antedating affidavit of the inventors shows more of the invention than what was disclosed by the reference, thereby satisfying the longstanding requirement set forth in *Clarke*. *Id.* Furthermore, there is nothing to indicate that the results shown in the declaration were limited to the exemplified species, rather it is apparent that the experiments were conducted as proof of the generic concepts which underlie the claimed invention. As such, the successful making and using of the designed constructs represents a completion of the invention prior to the earliest possible disclosure by Fire et al.

The declaration of the inventors proves that the inventors actually reduced the present invention to practice prior to the earliest possible effective filing date of Fire et al. Therefore, the reference is not a prior art reference against the current claims of the application. This reason alone is sufficient to overturn the rejection.

**(b) Fire et al., Brown et al., Lusky et al., Schiedner et al., and Baracchini et al. Could Not Have Rendered the Invention Obvious**

Among the distinctions between Fire et al. and the presently claimed inventions, the Examiner has acknowledged that Fire et al. do not teach an intron to be interposed between the sense and antisense strand of the chimeric construct. However, the Office ascribes more

to Fire et al. than is actually taught by the reference. Fire et al. teach the introduction of double stranded RNA (dsRNA) comprising two separate strands into *Caenorhabditis* to achieve gene silencing of target genes. At most, Fire et al. suggested that the dsRNA sequences could be present in an RNA molecule that could be expressed from a single gene. However, contrary to the allegation of the Examiner, Fire et al. are completely silent about the possibility of such a gene containing an intron sequence. Even the passages of Fire et al. cited by the Examiner on page 4 of the Official Action dated march 11, 2005 that do mention the word “intron” do so only in a completely different context. For example, at column 5, lines 40-4, Fire et al. indicated that targeted genes can contain exons and introns, not that an expressed dsRNA can contain an intron. In fact, at column 17, lines 20-25, Fire et al. teach that dsRNA segments matching intron sequences in target genes did not produce detectable inhibition. Given the results reported by Fire et al., one would be lead away from including intron sequences in a dsRNA. There is no suggestion or reason that may be derived from Fire et al. for one to include intron sequence in a dsRNA construct. Indeed, the Examiner’s position is in contradiction to the correct findings indicated in the Office Action mailed April 9, 2003 at 7.

“US Patent No 6506559 (Fire et al) is considered to be pertinent to Applicant’s disclosure because it discloses methods of inhibiting the expression of a target gene using a dsRNA molecule, including an RNA molecule expressed from a DNA vector, **however, Fire et al. does not teach these methods wherein the DNA vector comprises a heterologous intron, nor is there any suggestion in the prior art to include such an intron in the DNA vectors expressing a dsRNA of Fire et al.**”

OFFICE ACTION mailed April 9, 2003 at 7 (emphasis added).

Brown et al. does not teach or suggest a construct expressing a dsRNA and comprising a heterologous intron. Therefore, Brown et al. could not provide any direct reason to modify Fire et al. as the Examiner has proposed.

Consideration of the teachings and motivation of Brown et al. demonstrate that no reason to modify Fire et al. can be derived from Brown et al. Brown et al. is specifically directed at inclusion of particular intron sequences into the non-translated leader of a chimeric protein expression gene. In direct contradiction to the aim of the present invention, the aim of Brown et al. is to express greater quantities of proteins in plants. See, Brown et al. at column 1, lines 10-12. The presently claimed chimeric constructs are not directed to protein expression. There would have been no reason for a person of ordinary skill to

contemplate that whatever mechanism provides the increased protein expression observed by Brown et al. would have any affect on gene silencing. Thus a person of ordinary skill in the art would not find the teaching of Brown et al. to be relevant to the present invention.

There is no indication in Brown et al. that the observed increased protein expression was due to any effect that would be relevant to the present invention. The biologically active molecule for the reduction of the expression of the target gene in the current application is the transcribed RNA which can form an artificial hairpin double stranded RNA molecule, no protein is expressed from the claimed chimeric gene. Thus, the increase in protein expression observed by Brown et al. would have no relevance to the presently claimed invention and would provide no motivation to combine the references as proposed by the Office.

To the extent that one might have speculated that the increased protein expression of Brown et al. was related to increased transcription of RNA, there is also no indication in Fire et al. that increased transcription of a chimeric gene encoding a dsRNA molecule would lead to increased silencing of the target gene. To the contrary, what Fire et al. taught is that low concentrations of RNA molecules are effective for dsRNA mediated gene silencing (column 5, lines 15 to 17). In other words, there is no indication that higher concentrations of dsRNA would produce a greater gene silencing. Thus, there is no incentive provided by the Fire et al. disclosure to increase the expression of a gene encoding a dsRNA molecule to improve the silencing of the expression of the target gene, even if Brown et al. could be read to suggest that an intron might increase production of RNA, which it cannot.

Lusky et al. is cited for its alleged teaching of expression constructs comprising antisense RNA and for teaching that expression construct can comprise an intron as well as other expression elements including translation termination and polyadenylation signals. Lusky et al. provides teaching concerning the construction of viral expression vectors and helper cells. Genes in these constructs may contain intron sequences just as many genes include introns. Schnieder et al. teach expression vectors comprising intronic sequences for enhancing vector stability. In constructing viral vectors, intron sequences may be included to match the size of the vector to the capacity of the viral packaging for improved production of viral particles. These teachings are simply not relevant to the construction of the chimeric genes of the invention. The teachings of Lusky et al. and Schiedner et al. provide no reason for a person of ordinary skill to modify a chimeric gene encoding an artificial hairpin dsDNA to include an intron.

Fire et al. in column 3, lines 19-34 states that “inhibition by dsRNA must occur by a mechanism distinct from antisense interference”. Therefore, at the time the invention was made, the person skilled in the art would not have extrapolated any suggestions regarding antisense constructs that might be ascribed to Lusky et al. to be to be relevant to dsRNA mediated gene silencing. Even if vectors existed in the prior art containing heterologous DNA with intron sequences, they have no apparent relevance to improving dsRNA silencing, and certainly do not provide any reason for a person of ordinary skill in the art to include an intron in a chimeric artificial hairpin RNA as in the present claims.

Baracchini et al. is cited for its teaching of the ability to target a gene of interest with a complementary sequence comprising at least 10 nucleobases. Baracchini et al. was cited only for allegedly teaching sizes and amounts of sequence identity between the complementary RNA and the target gene to be silenced. Baracchini et al. fails to remedy the deficiencies of the combination of Fire et al. with Brown et al. and Lusky et al.

**(c) Inclusion of an Intron Produces Surprising Unpredicted Improvement in the Invention**

As disclosed in the present application, the inventors discovered that inclusion of an intron in a hairpin dsRNA gene silencing construct provides surprisingly improved gene silencing. These findings were published in the scientific literature by the inventors in Smith et al., *Nature*, 407:319-32, 2000 (EXHIBIT 9). The improved efficiency provided by the inclusion of an intron in the construct was not predicted or predictable. However, the improvement has been widely adopted in the art since the publication of Smith et al.. As may be seen from the sample of papers that Appellants have provided to the Examiner, persons of ordinary skill in the art have widely adopted the use of hairpin dsRNA constructs comprising introns and have cited the work of the inventors in numerous reports. *See* Exhibits 10-18.

Even when the phenomenon of RNA interference by dsRNA was published, persons of ordinary skill in the art did not know the mechanism of the effect. Wagner and Sun, 1998, *Nature*, 39:744-45 (EXHIBIT 24). Therefore, no one could have predicted how the effect might have been improved. The Smith et al. report explained that presence of an intron enhances silencing efficiency by either

- a. increased formation of duplex RNA during the process of intron excision from the construct by the spliceosome due to better alignment of the complementary arms of the hairpin in an environment favoring RNA hybridization;

- b. increased retention of hairpin RNA in nucleus; or
- c. creation of a smaller, less nuclease-sensitive loop.

Smith et al. at 320, paragraph spanning the 1<sup>st</sup> and 2<sup>nd</sup> column).

None of Fire et al., Brown et al., Lusky et al., Schiedner et al., or Baracchini et al. predicted or provided for any of these potential actions attached to the inclusion of an intron in a chimeric hairpin dsRNA. Thus, neither any of these references, nor the state of the art as a whole could have predicted the effect that was first disclosed by the inventors in the present application. The of surprising results, lack of predictability, and widespread adoption of the invention following its disclosure are all secondary considerations pointing to the fact that the invention was not obvious. *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1, 15-17 (1966).

**(d) The Rejection Over Fire et al., Brown et al., Lusky et al., Schiedner et al., and Baracchini et al. Must Be Overturned**

As explained above, Fire et al. is not prior art to the present application. Thus, the basis of the rejection is fatally flawed and it cannot be sustained.

Furthermore, even if Fire et al. were prior art, because none of Brown et al., Lusky et al., Schiedner et al., and Baracchini et al. provides a cognizable reason for a person of ordinary skill to have included an intron in the presently claimed constructs, the combination of references could not have rendered the invention obvious.

The inclusion of an intron in the claimed chimeric constructs has been shown to produce a surprising and unpredicted improvement in the effect of the invention that has led to widespread adoption of the invention in the art. Thus, there are strong secondary considerations that point inexorably toward the conclusion that the invention could not have been obvious at the time it was made.

**2. Rejection over Flavell, Metzloff et al., Stam et al., Brown et al. and Lusky et al.**

Claims 22, 26, 42, 53-54, 56, 58, 63-69, 100-103, 109, and 115-122 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Flavell (Proc. Natl. Acad. Sci., 91:3490-96, 1994) (EXHIBIT 6), Metzloff et al. (Cell, 88:845-54, 1997) (EXHIBIT 2) and Stam et al. (Annals of Botany, 79:3-12, 1997) (EXHIBIT 3), the combination in view of Brown et al., (US 5,859,347) (EXHIBIT 4), and Lusky et al. (US 6,350,575) (EXHIBIT 5).

The Examiner alleges Flavell et al. discloses plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation region and further comprising a DNA region encoding a region capable of forming a double stranded RNA stem by base pairing between regions with a sense and antisense nucleotide sequence, which sense nucleotide sequence includes at least 10 consecutive nucleotides having 100% sequence identity with at least 10 consecutive nucleotides having 100% sequence identity with said at least 10 consecutive nucleotides of the sense sequence. *See* OFFICE ACTION mailed September 19, 2008 at 6.

The Examiner further contends that Metzloff et al. teaches plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation regions, and further comprising a DNA region encoding a region capable of forming a double stranded RNA by base pairing between regions with a sense and an antisense nucleotide sequence. *See* OFFICE ACTION mailed September 19, 2008 at 7.

The Examiner further alleges that Stam et al. discloses plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation region and further comprising a DNA region encoding a region capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base pairing between regions with a sense and antisense nucleotide sequence, which sense nucleotide sequence includes at least 10 consecutive nucleotides having 100% sequence identity with at least 10 consecutive nucleotides having 100% sequence identity with said at least 10 consecutive nucleotides of the sense sequence. *See* OFFICE ACTION mailed September 19, 2008 at 7.

Brown et al. and Lusky et al. have been cited as teaching the inclusion of intron sequences in chimeric gene vectors as described above.

**(a) The Examiner's Hindsight Review is Contradicted by the Objective Response of the Scientific Community to the Invention as Evidenced by Nobel Prize Committee**

The Examiner admits that none of Flavell, Metzloff et al. and Stam et al. teach double stranded hairpin constructs in their inverted repeats, nor do they teach the insertion of an intron in their double stranded inhibitory constructs. *See* OFFICE ACTION mailed September 19, 2008 at 7, last paragraph.

However, the Examiner contends that it would have been obvious to one of ordinary skill in the art at the time of the invention was made to alter the expression of a target gene of known sequence comprising the introduction of nucleic acids comprising sense and complimentary antisense sequences of the target genes operably linked to a promoter and which are optionally expressed on separate or the same expression construct, because this approach to gene silencing had been proposed and studied previously by Flavell, Metzlaff et al. and Stam et al. *See* OFFICE ACTION mailed September 19, 2008 at 8.

To remedy the admitted deficiency of the primary references, the Examiner has alleged that one of ordinary skill in the art would have been motivated to design inverted repeats in a single molecule to test its inhibitory capacity because expression of a single, contiguous self annealing construct would provide for more efficient self annealing compared to two separately expressed self annealing molecules, applying scientific logic to the teachings of Flavell, Metzlaff and Stam. *See* OFFICE ACTION mailed September 19, 2008 at 9, lines 3-9.

The Examiner's position is essentially that a person of ordinary skill in the art reading Flavell, Metzlaff et al. and Stam et al. would have found it obvious to make not only the dsRNA of Fire et al., which is contrary to the issuance of Fire et al. U.S. Patent No. 6,506,559, but, then to further go on to manufacture the dsRNA gene silencing constructs comprising an artificial hairpin of sense and antisense gene targeting sequences and an intron. That is, the Examiner has contended that Flavell, Metzlaff et al. and Stam et al. not only rendered the phenomenon of RNA interference obvious, but also the further improvements that have been disclosed in the present application.

The Examiner's interpretation of the state of the art is contradicted by the response of the scientific community to the first publication describing the discovery of the RNA interference phenomenon. The first publication describing the RNA interference phenomenon earned the Nobel prize for its authors. The announcement of the 2006 Nobel Prize in Physiology or Medicine states:

Fire and Mello published their findings in the journal Nature on February 19, 1998. Their discovery clarified many confusing and contradictory experimental observations and revealed a natural mechanism for controlling the flow of genetic information. This heralded the start of a new research field.

Nobelprize.org: The Nobel Prize in Physiology or Medicine 2006, Press Release of the Nobel Assembly at Karolinska Institute (October 2, 2006)(EXHIBIT 26); *see also* Fire A., Xu S.Q.,

Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C., 1998, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391:806-11 (EXHIBIT 26).

As explained above, the Declaration of the Inventors under 37 C.F.R. § 1.131 (EXHIBIT 23) proves that the present invention was actually reduced to practice prior to the filing of the Fire et al. patent application and the subsequent publication of their results. The award of a Nobel Prize is perhaps the greatest recognition that the scientific community can offer to a novel and surprising discovery. Indeed in commentary published in the same issue as the Fire et al. *Nature* publication, Wagner and Sun described the reported effect of dsRNA targeting genes in *C. elegans* as “remarkable and surprising.” Wagner and Sun, *Nature*, 39:744-45 (1998) (EXHIBIT 24). Wagner and Sun provide further contemporaneous evidence that the phenomenon of RNA interference which underlies the present invention was not obvious even to those of extraordinary skill in the art at the time the invention was made. Therefore, the present invention, which was completed before the first publication reporting RNA interference could not possibly have been obvious.

The Examiner’s reading of the references appears to be influenced by hindsight consideration of knowledge that was disclosed only after the present invention was made. However, the objective evidence of the history of RNA interference proves that at the time that the invention was made persons of ordinary skill in the art did not and could not have anticipated the surprising effects of RNA interference using dsRNA nor could they have predicted that RNA interference would even work let alone that the phenomenon could be improved by the invention claimed in the present application.

**(b) Dr. Metzloff’s Testimony Contradicts the Examiner’s Interpretation of Flavell, Metzloff et al. and Stam et al.**

Appellants also presented a declaration by Dr Michael Metzloff pursuant to 37 C.F.R. § 1.132 (EXHIBIT 22). Dr. Metzloff who was an expert in the field of gene-silencing at the time the invention was made and who is co-author of one of the primary references relied on by the Examiner contradicts the Examiner’s interpretation of the state of the art at the time the invention was made. Dr. Metzloff provides his testimony concerning what the references would have taught a person of ordinary skill in the art given the general state of the art the time the invention was made.



Dr Michael Metzloff testifies that the publications by Flavell, Metzloff et al. and Stam et al (or other contemporaneous publications related to the field of co-suppression) did not contemplate that double stranded RNA structures formed between antisense RNA and the sense mRNA could be a triggering agent in gene silencing. Declaration of Metzloff at ¶ 9. Dr Metzloff further testifies that at that point in time the favorite hypothesis to explain the co-suppression mechanism of action included the model that co-suppression was mediated through the involvement of an antisense molecule generated via a unknown mechanism from the sense RNA. The antisense RNA molecule could then form a dsRNA intermediate with the targeted mRNAs which were thus tagged for degradation. DECLARATION OF METZLOFF at ¶ 14. According to this model, the antisense RNA is the pivotal molecule in gene-silencing.

Dr Metzloff also declares that a person of ordinary skill in the art understanding the proposed models for gene silencing would not have included a sense and antisense RNA strand in one single molecule to obtain more efficient self annealing. This is because the proposed models and prevailing wisdom considered a antisense strand to be the operative gene-silencing triggering molecule. More efficient self annealing would sequester the antisense strand by base-pairing with the sense strand, which would be contrary to the mechanisms proposed in the cited papers and generally understood at the time. DECLARATION OF METZLOFF at ¶ 9. He also testifies that he was a person of at least ordinary skill at the time and certainly would never have contemplated deliberately introducing complimentary sense and antisense sequence of a target gene which can form a double stranded RNA molecule to increase the efficiency of gene silencing based upon these papers or the general understanding in the art at the time. DECLARATION OF METZLOFF at ¶ 9.

Dr Metzloff concludes that Flavell, Stam and Metzloff all emphasize the importance of the complementary RNA/antisense RNA as the central effector molecule in gene silencing and reiterates that accordingly it would not be logical to enhance the efficiency of gene silencing by simultaneous introduction of a sense and antisense RNA molecule capable of forming a duplex RNA with each other, since this sense RNA molecule would compete with the targeted mRNA molecule for duplex formation with the active antisense molecule triggering the gene-silencing phenomenon. The competition would even be more severe if the introduced sense and antisense RNA would be present in one molecule, as such intramolecular duplex formation would be favored over intermolecular duplex formation. DECLARATION OF METZLOFF at ¶ 31. Therefore, Dr Metzloff expresses his opinion that it would **not** have been obvious to one of ordinary skill in the art to derive from the Flavell,

Metzlaff et al. and Stam et al. publications (or other contemporaneous publications in the field of co-suppression in plants) that expression of target genes in a cell can be inhibited by the introduction of chimeric genes expressing sense and complimentary antisense sequences of the target gene (either from separate constructs or from the same construct) which can form a double stranded RNA molecule. DECLARATION OF METZLAFF at ¶ 32.

Dr Metzlaff's declaration thus refutes the Examiner's reading of the primary references as teaching modification of the expression of a target gene of known sequence comprising the introduction of nucleic acids comprising sense and complimentary antisense sequences of the target genes operably linked to a promoter and which are optionally expressed on separate or the same expression construct. Furthermore, Dr Metzlaff's declaration traverses the Examiner's opinion that a person of ordinary skill in the art understanding the proposed models for gene silencing would have included a sense and antisense RNA strand in one single molecule to obtain more efficient self annealing.

The secondary references by Brown et al. and Lusky et al. are only relied upon to demonstrate that in certain circumstances introns had been used in expression constructs in the prior art. However, since the secondary references are silent with regard to teaching modification of the expression of a target gene of known sequence comprising the introduction of nucleic acids comprising sense and complimentary antisense sequences of the target genes operably linked to a promoter, they could not remedy the deficiencies of Flavell, Stam et al. and Metzlaff et al.. Moreover, the inaptness of these references has been previously addressed above.

**(c) The Rejection over Flavell, Metzlaff et al., Stam et al.,  
Brown et al. and Lusky et al. Must Be Overturned**

For at least the reasons elaborated herein, the currently claimed invention would not have been obvious over Flavell, Metzlaff et al. and Stam et al., the combination in view of Brown et al. Lusky et al.

Appellants have shown through objective evidence that those of skill in the art considered the underlying discovery of RNA interference using dsRNA to be surprising and remarkable at the time it was first described, so much so that the first authors to publish on the subject were awarded the Nobel Prize. Appellants have demonstrated through affidavit that the present invention was completed prior to that first publication by Fire et al. or the filing of a patent application by Fire et al.

Appellants have further proven through testimonial evidence that the Examiner's hindsight consideration of the state of the art does not accurately reflect the understanding of a person of ordinary skill at the time the invention was made. Rather, the prior art would not, could not, and did not render the invention obvious to even an expert in the field (and an author of the cited prior art) at the time the invention was made.

Accordingly, the rejection must be overturned and such action is respectfully requested.

### **C. Double Patenting**

Claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109, and 115-122 stand provisionally rejected under the doctrine of obviousness type double patenting over pending claims 22-29, 35-38 of U.S. Patent Application No. 11/841,737. Appellants note that claims 22-29 have been canceled in U.S. Patent Application No. 11/847,737.

The Examiner has acknowledged that the claims under appeal are not identical to the cited claims. However, the Examiner has failed to accurately identify the differences between the cited claims and the rejected claims and to explain why those differences would be obvious. Such an analysis is essential to a legal determination of obviousness. *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1 (1966).

The claims in the present application recite features, such as the inclusion of an intron in the construct, that are not recited in claims 35-38 of U.S. Patent Application No. 11/841,737. As explained above, the secondary references that the Examiner has cited for the intron feature do not provide any relevant reason to include an intron in the claimed chimeric gene constructs and cells. Moreover, the pending claims of U.S. Patent Application No. 11/841,737 recite features that would not be obvious in view of the present claims. Appellants respectfully submit that in the absence of a cognizable reason to modify the claims of the '737 application to contain the features of the present claims, the Examiner has failed to establish a prima facie case of obviousness. Furthermore, the present application was filed prior to U.S. Patent Application No. 11/841,737. Therefore it would be appropriate for the Office to withdraw the provisional rejection and allow the present application to issue.

Therefore, the rejection should be overturned and such action is respectfully requested.

**D. Conclusion**

In view of the forgoing, each of the rejections on appeal should be overturned. Appellants respectfully request that the Board issue an order overturning each of the rejections on appeal and directing that the application be allowed to issue.

**VIII. Claims Appendix**

See attached Claims Appendix for a copy of the claims involved in the appeal.

**IX. Evidence Appendix**

See attached Evidence Appendix for copies of evidence relied upon by Appellant.

**X. Related Proceedings Appendix**

See attached Related Proceedings Appendix for copies of decisions identified in Section II, supra.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date April 8, 2010

By: /Christopher L North/  
Christopher L. North  
Registration No. 50433

**Customer No. 21839**  
703 836 6620

## VIII. CLAIMS APPENDIX

### The Appealed Claims

A listing of all the pending claims is presented. Claims 22, 26, 42, 53, 54, 56, 58, 63-69, 100-103, 109 and 115-122 stand rejected and are the subject of this appeal.

Claim 1 (Withdrawn) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, comprising the step of introducing into said plant cell a chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in said plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences,
  - wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence that is identical to at least 20 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest,
  - and wherein the second of said annealing RNA sequences comprises an antisense sequence that is identical to at least 20 consecutive nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest,
  - and wherein said DNA region comprises an intron heterologous to said sense sequence; and
- c) a DNA region involved in transcription termination and polyadenylation.

Claim 2 (Withdrawn) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, comprising the step of introducing into said plant cell a chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in said plant cell;

b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising

i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest; and

ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,

and wherein said DNA region comprises an intron heterologous to said sense nucleotide sequence; and

c) a DNA region involved in transcription termination and polyadenylation.

Claim 3 (Withdrawn) The method of claim 2, wherein said RNA molecule further comprises a spacer nucleotide sequence located between said sense and said antisense nucleotide sequence.

Claim 4 (Withdrawn) The method of claim 2, wherein said sense nucleotide sequence comprises at least about 550 consecutive nucleotides having 100% sequence identity with at least about 550 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest.

Claim 5 (Withdrawn) The method of claim 2, wherein said nucleic acid of interest is a gene integrated in the genome of said plant cell.

Claim 6 (Previously Presented) The method of claim 5, wherein said gene is an endogenous gene.

Claim 7 (Withdrawn) The method of claim 5, wherein said gene is a foreign transgene.

Claim 8 (Withdrawn) The method of claim 2, wherein said chimeric DNA is stably integrated in the genome of said plant cell.

Claim 9 (Withdrawn) The method of claim 2, wherein said nucleic acid of interest is comprised in the genome of an infecting virus.

Claim 10 (Withdrawn) The method of claim 9, wherein said infecting virus is an RNA virus.

Claim 11 (Canceled).

Claim 12 (Withdrawn) The method of claim 2, wherein said plant cell is comprised within a plant.

Claims 13-21 (Canceled).

Claim 22 (Previously Presented) A plant cell, comprising a nucleic acid of interest, which is normally capable of being phenotypically expressed, further comprising a chimeric DNA molecule comprising the following operably linked parts:

- a) a promoter, operative in said plant cell;

b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising

i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of the nucleic acid of interest; and

ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence,

and wherein said DNA region comprises an intron heterologous to said sense nucleotide sequence; and

c) a DNA region involved in transcription termination and polyadenylation.

Claims 23-25 (Canceled).

Claim 26 (Previously presented) A plant comprising the plant cell of claim 22.

Claims 27-39 (Canceled).

Claim 40 (Withdrawn) The method of claim 2, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.



Claim 41 (Canceled).

Claim 42 (Previously presented) The plant cell of claim 22, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 43 (Withdrawn) The method of claim 2, wherein said sense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with at least 50 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with the complement of said at least 50 consecutive nucleotides of said sense nucleotide sequence.

Claim 44 (Previously Presented) The method of claim 2, wherein said sense nucleotide sequence includes at least 100 consecutive nucleotides having 100% sequence identity with at least 100 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 100 consecutive nucleotides having 100% sequence identity with the complement of said at least 100 consecutive nucleotides of said sense nucleotide sequence.

Claim 45 (Canceled).

Claim 46 (Previously Presented) The method of claim 43 wherein said intron is located between the DNA region encoding said sense nucleotide sequence and the DNA region encoding said antisense nucleotide sequence.

Claims 47-49 (Canceled).

Claim 50 (Withdrawn) The method of claim 44, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claims 51-52 (Canceled).

Claim 53 (Previously Presented) The plant cell of claim 22, wherein said sense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with at least 50 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with the complement of said at least 50 consecutive nucleotides of said sense nucleotide sequence.

Claim 54 (Previously Presented) The plant cell of claim 22, wherein said sense nucleotide sequence includes at least 100 consecutive nucleotides having 100% sequence identity with at least 100 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 100 consecutive nucleotides having 100% sequence identity with the complement of said at least 100 consecutive nucleotides of said sense nucleotide sequence.

Claim 55 (Canceled).

Claim 56 (Previously Presented) The plant cell of claim 53, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 57 (Canceled).

Claim 58 (Previously Presented) The plant cell of claim 54, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claims 59-62 (Canceled).

Claim 63 (Previously Presented) A chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in a plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences,
  - wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest,
  - and wherein the second of said annealing RNA sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest,
  - and wherein said DNA region comprises an intron heterologous to said sense sequence; and
- c) a DNA region involved in transcription termination and polyadenylation.

Claim 64 (Previously Presented) A chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in a plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising

i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest; and

ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,

wherein said DNA region comprises an intron heterologous to said region with sense nucleotide sequence; and

c) a DNA region involved in transcription termination and polyadenylation.

Claim 65 (Previously presented) The chimeric DNA of claim 64, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 66 (Previously Presented) The chimeric DNA of claim 64, wherein said sense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with at least 50 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with the complement of said at least 50 consecutive nucleotides of said sense nucleotide sequence.

Claim 67 (Previously Presented) The chimeric DNA of claim 64, wherein said sense nucleotide sequence includes at least 100 consecutive nucleotides having 100% sequence identity with at least 100 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 100 consecutive nucleotides having 100% sequence identity with the complement of said at least 100 consecutive nucleotides of said sense nucleotide sequence.

Claim 68 (Previously presented) The chimeric DNA of claim 66, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 69 (Previously presented) The chimeric DNA of claim 67, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claims 70-97 (Canceled).

Claim 98 (Withdrawn) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, comprising the step of introducing into said plant cell a chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in said plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences,  
wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest,

and wherein the second of said annealing RNA sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest,

and wherein said DNA region comprises an intron ; and

c) a DNA region involved in transcription termination and polyadenylation.

Claim 99 (Withdrawn) A method for reducing the phenotypic expression of a nucleic acid of interest, which is normally capable of being expressed in a plant cell, comprising the step of introducing into said plant cell, a chimeric DNA comprising the following operably linked parts:

a) a promoter, operative in said plant cell;

b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising

i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest; and

ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,

and wherein said DNA region comprises an intron ; and

c) a DNA region involved in transcription termination and polyadenylation.

Claim 100 (Previously Presented) A plant cell, comprising a nucleic acid of interest, which is normally capable of being phenotypically expressed, further comprising a chimeric DNA molecule comprising the following operably linked parts:

- a) a promoter, operative in said plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of the nucleic acid of interest; and
  - ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence,

and wherein said DNA region comprises an intron ; and

- c) a DNA region involved in transcription termination and polyadenylation.

Claim 101 (Previously presented) A plant comprising the plant cell of claim 100.

Claim 102 (Previously Presented) A chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in a plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences,

wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest, and wherein the second of said annealing RNA sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of at least part of said nucleotide sequence of said nucleic acid of interest, and wherein said DNA region comprises an intron; and

c) a DNA region involved in transcription termination and polyadenylation.

Claim 103 (Previously Presented) A chimeric DNA comprising the following operably linked parts:

- a) a promoter, operative in a plant cell;
- b) a DNA region, which when transcribed, yields an RNA molecule with a nucleotide sequence comprising
  - i) a sense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest; and
  - ii) an antisense nucleotide sequence including at least 20 consecutive nucleotides having 100% sequence identity with the complement of said at least 20 consecutive nucleotides of said sense nucleotide sequence;

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence such that said at least 20 consecutive nucleotides of the sense sequence basepair with said at least 20 consecutive nucleotides of the antisense sequence,

and wherein said DNA region comprises an intron ; and

c) a DNA region involved in transcription termination and polyadenylation.



Claims 104-108 (Canceled).

Claim 109 (Previously Presented) The chimeric DNA of claim 64, wherein said RNA molecule further comprises a spacer nucleotide sequence located between said sense and said antisense nucleotide sequence.

Claim 110 (Canceled).

Claim 111 (Withdrawn) The method of claim 99, wherein said RNA molecule further comprises a spacer nucleotide sequence located between said sense and said antisense nucleotide sequences.

Claim 112 (Withdrawn) The method of claim 99, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 113 (Withdrawn) The method of claim 99, wherein said sense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with at least 50 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with the complement of said at least 50 consecutive nucleotides of said sense nucleotide sequence.

Claim 114 (Withdrawn) The method of claim 113, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 115 (Previously Presented) The plant cell of claim 100, wherein said RNA molecule further comprises a spacer nucleotide sequence located between said sense and said antisense nucleotide sequences.

Claim 116 (Previously Presented) The plant cell of claim 100, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 117 (Previously Presented) The plant cell of claim 100, wherein said sense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with at least 50 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with the complement of said at least 50 consecutive nucleotides of said sense nucleotide sequence.

Claim 118 (Previously Presented) The plant cell of claim 117, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 119 (Previously Presented) The chimeric DNA of claim 103, wherein said RNA molecule further comprises a spacer nucleotide sequence located between said sense and said antisense nucleotide sequences.

Claim 120 (Previously Presented) The chimeric DNA of claim 103, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claim 121 (Previously Presented) The chimeric DNA of claim 103, wherein said sense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with at least 50 consecutive nucleotides of the nucleotide sequence of said nucleic acid of interest, and said antisense nucleotide sequence includes at least 50 consecutive nucleotides having 100% sequence identity with the complement of said at least 50 consecutive nucleotides of said sense nucleotide sequence.

Claim 122 (Previously Presented) The chimeric DNA of claim 121, wherein said intron is located between part of said DNA region which when transcribed yields said sense nucleotide sequence and part of said DNA region which when transcribed yields said antisense nucleotide sequence.

Claims 123-134 (Canceled).

## IX. EVIDENCE APPENDIX

Exhibit #	Description	Where entered in the record.
1	Fire et al. (US 6,506,559)	PTO-892 November 1, 2007
2	Metzlaff et al. (Cell, 88:845-54, 1997)	PTO-892 November 1, 2007
3	Stam et al. (Annals of Botany, 79:3-12, 1997)	PTO-892 November 1, 2007
4	Brown et al., (US 5,859,347)	PTO-892 November 1, 2007
5	Lusky et al. (US 6,350,575)	PTO-892 November 1, 2007
6	Flavell (Proc. Natl. Acad. Sci., 91:3490-96, 1994)	PTO-892 November 1, 2007
7	Schiedner et al. (Nature Genetics, 18:180-83, 1998)	PTO-892 November 1, 2007
8	Baracchini et al. (US 5,801,154)	PTO-892 November 1, 2007
9	Smith et al. 2000, <i>Nature</i> , 407, 319-320	IDS filed November 22, 2006
10	Wesley et al. 2001, <i>The Plant Journal</i> , 27:581-90	IDS filed November 22, 2006
11	Samuel and Ellis 2002, <i>The Plant Cell</i> , 14: 2059-69	IDS filed November 22, 2006
12	Acosta-Garcia and Vielle-Calzada 2004 - <i>The Plant Cell</i> , 16: 2614-28	IDS filed November 22, 2006
13	Guo et al., 2003, <i>The Plant Journal</i> , 34: 383-92	IDS filed November 22, 2006
14	Chen et al., 2003, <i>The Plant Journal</i> , 36: 731-40	IDS filed November 22, 2006

<b>Exhibit #</b>	<b>Description</b>	<b>Where entered in the record.</b>
15	Byzova et al., 2004, <i>Planta</i> , 218:379-87	IDS filed November 22, 2006
16	Lee et al., 2003, <i>Methods</i> , 30: 322-29	IDS filed November 22, 2006
17	Li et al., 2005, <i>The Plant Cell</i> , 17:859-75	IDS filed November 22, 2006
18	O'Brien et al. 2002, <i>The Plant Journal</i> , 32:985-96	IDS filed November 22, 2006
19	Declaration of Dr. Elizabeth Salisbury Dennis filed August 8, 2007, and attachments	Filed with Applicants' Reply dated August 8, 2007
20	Declaration of Dr. Marc de Block filed August 8, 2007, and attachments	Filed with Applicants' Reply dated August 8, 2007
21	Declaration of Dr Peter Schofield filed May 7, 2008, and attachments	Cited in Applicant's Amendment and Reply dated May 1, 2008 and re-filed in corrected form May 7, 2008
22	Declaration of Dr Michael Metzloff filed March 19, 2009, and attachments	Cited in Applicant's Amendment and Reply dated March 19, 2009
23	Declaration of the Inventors Under 37 C.F.R. § 1.131 filed May 1, 2008, and attachments	Filed with Applicants Amendment and Reply dated May 1, 2008
24	Wagner and Sun, 1998, <i>Nature</i> , 39:744-45	Filed as Annex III to the Declaration of Dr Michael Metzloff filed March 19, 2009.

<b>Exhibit #</b>	<b>Description</b>	<b>Where entered in the record.</b>
25	Nobelprize.org: The Nobel Prize in Physiology or Medicine 2006, Press Release of the Nobel Assembly at Karolinska Institute (October 2, 2006)	PTO-892 dated December 8, 2009
26	Fire A., Xu S.Q., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C., 1998, <i>Nature</i> , 391:806-11	IDS filed June 2, 2005



(12) **United States Patent**  
Fire et al.

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(45) Date of Patent: **\*Jan. 14, 2003**

(54) **GENETIC INHIBITION BY  
DOUBLE-STRANDED RNA**

(75) Inventors: **Andrew Fire**, Baltimore, MD (US);  
**Stephen Kostas**, Chicago, IL (US);  
**Mary Montgomery**, St. Paul, MN  
(US); **Lisa Timmons**, Lawrence, KS  
(US); **SiQun Xu**, Ballwin, MO (US);  
**Hiroaki Tabara**, Shizuoka (JP);  
**Samuel E. Driver**, Providence, RI  
(US); **Craig C. Mello**, Shrewsbury, MA  
(US)

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(73) Assignee: **Carnegie Institute of Washington**,  
Washington, DC (US)

(\*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(58) Field of Search ..... 514/44; 435/6,  
435/91.1, 325

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*Primary Examiner*—Andrew Wang

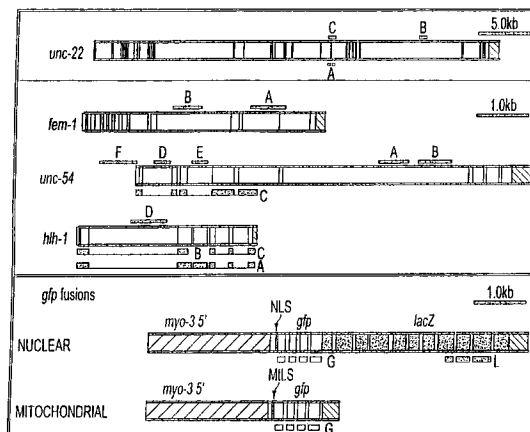
*Assistant Examiner*—Karen A Lacourciere

(74) *Attorney, Agent, or Firm*—Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced ex vivo or in vivo. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

**22 Claims, 5 Drawing Sheets**



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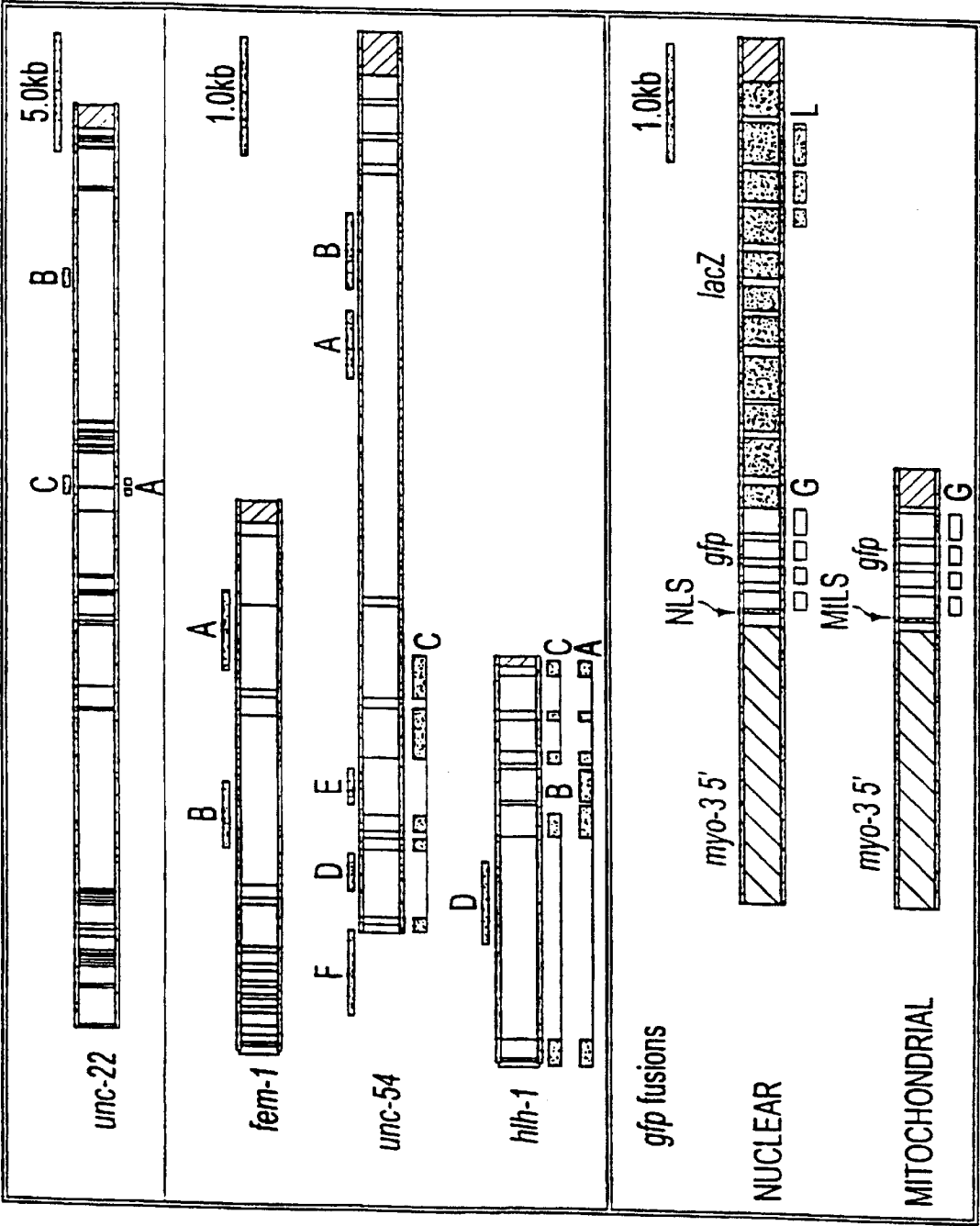
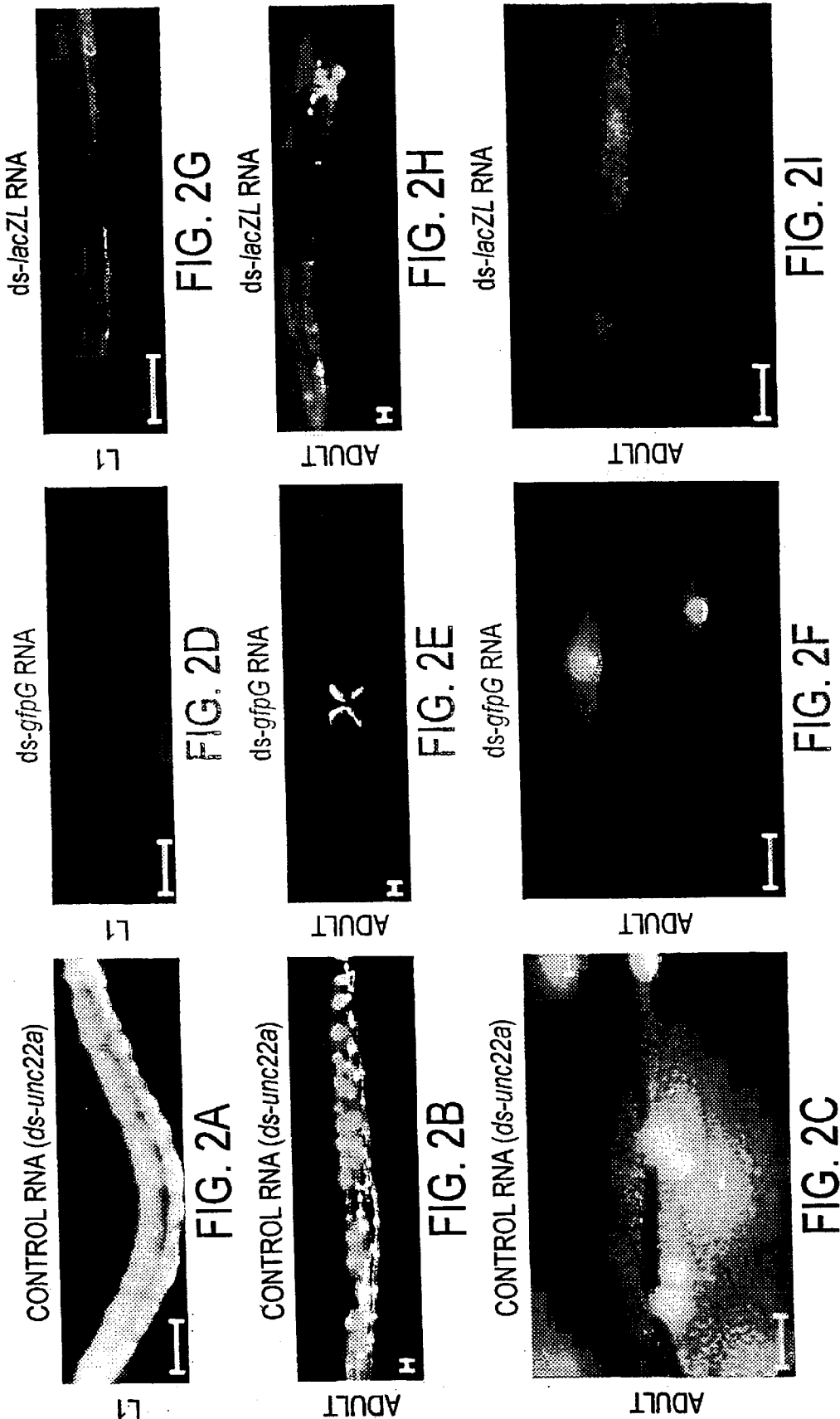


FIG. 1



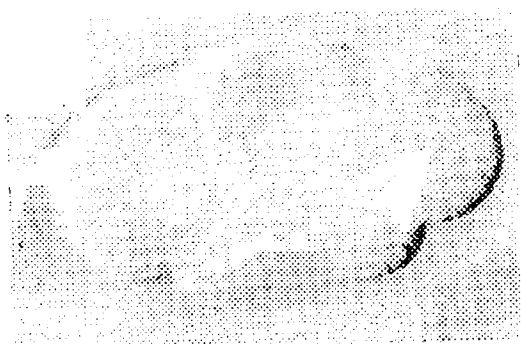


FIG. 3A



FIG. 3B

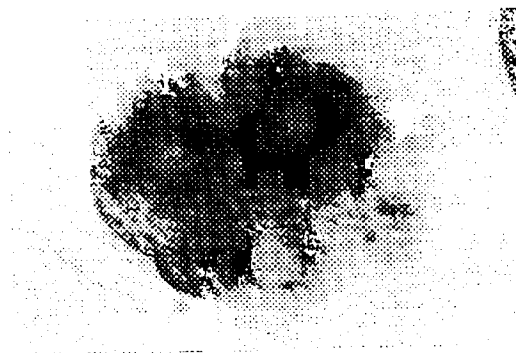


FIG. 3C

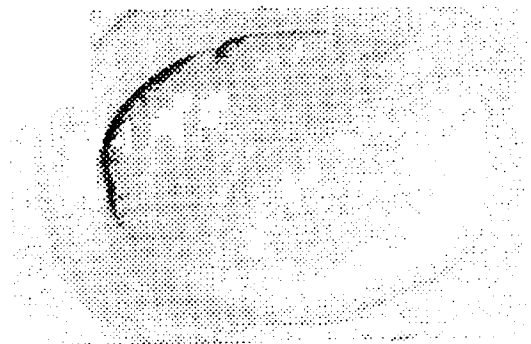


FIG. 3D

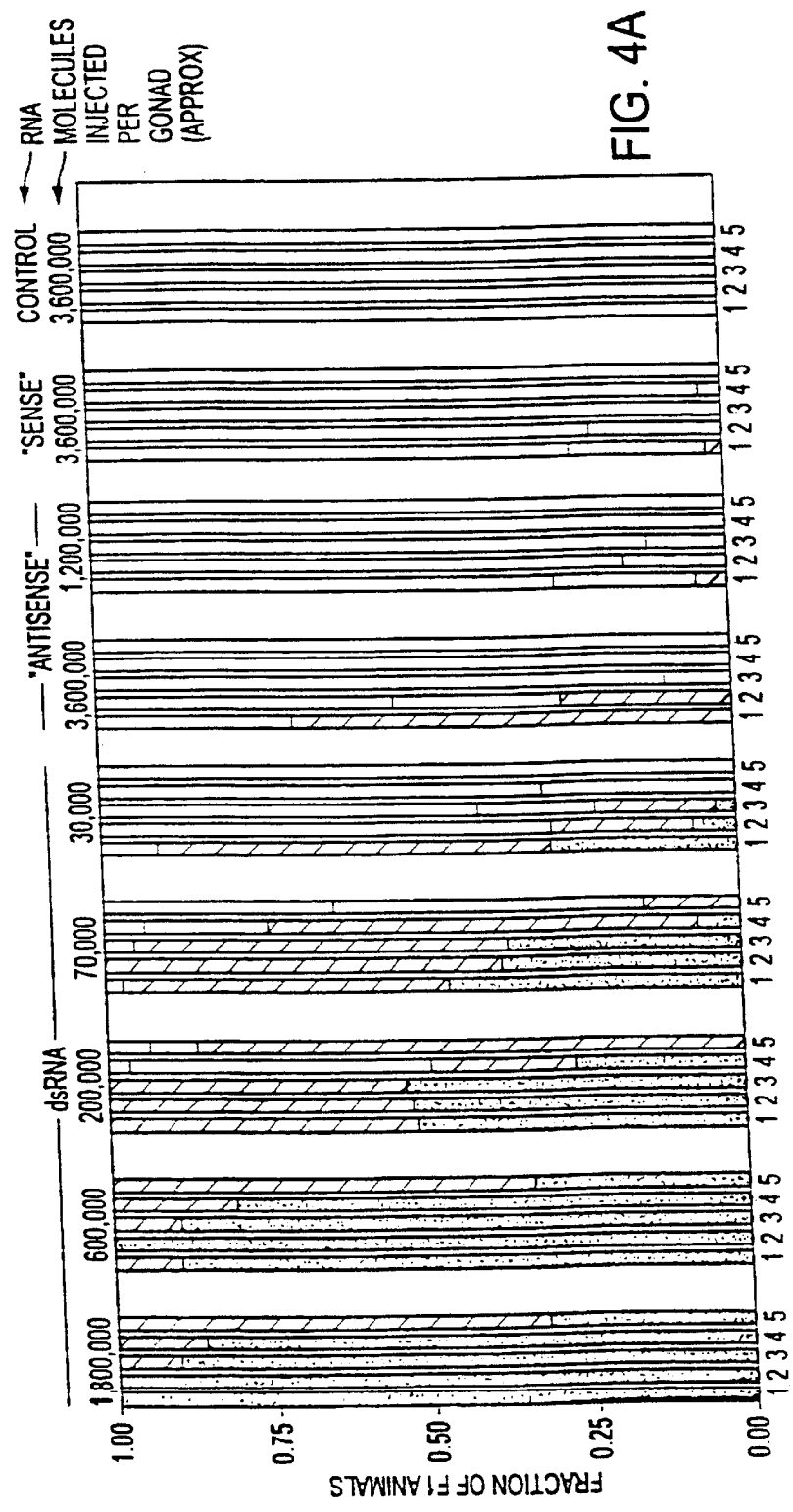


FIG. 4A

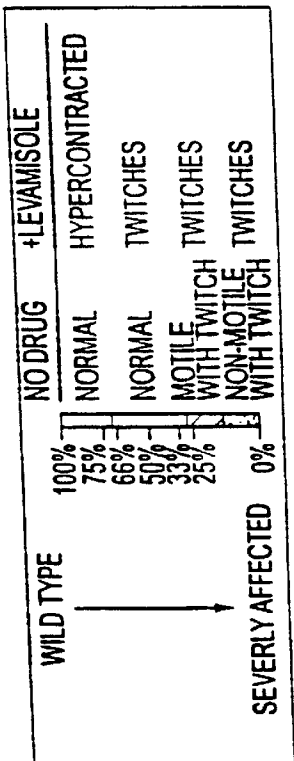


FIG. 4B

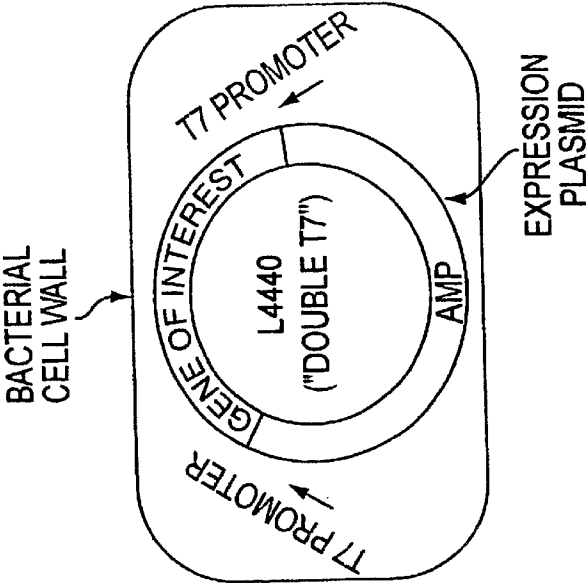


FIG. 5A

PD4251 WORMS

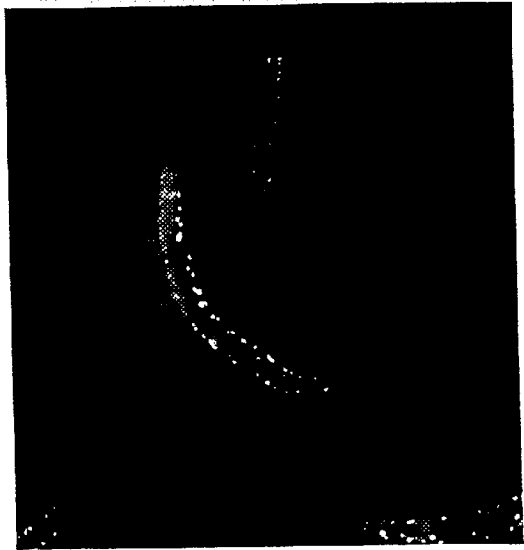


FIG. 5B

PD4251 WORMS FED BACTERIA  
EXPRESSING *gfp* dsRNA

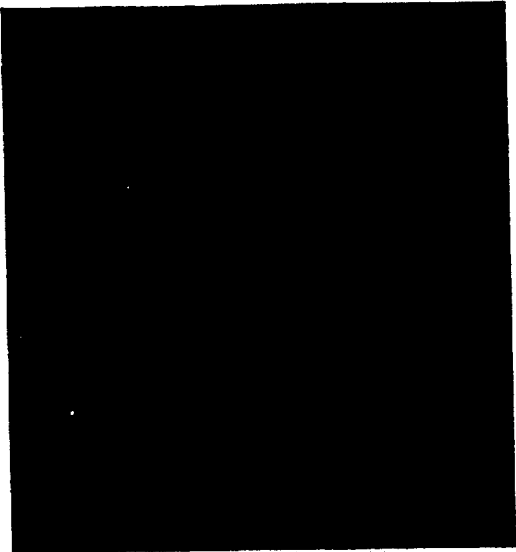


FIG. 5C

## GENETIC INHIBITION BY DOUBLE-STRANDED RNA

### RELATED APPLICATION

This application claims the benefit of U.S. Provisional Appln. No. 60/068,562, filed Dec. 23, 1997. +gi

### GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

#### 2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a directed change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which it is important to produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

#### Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stoichiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the

cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

#### Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule in vitro under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use in vivo has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells in vivo. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

#### Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been hypothesized to occur; an as-yet-unidentified mechanism would then lead to de novo methylation and subsequent suppression of gene expression.



Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

#### Distinction Between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

#### Distinction Between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition in *C. elegans* is mediated by a triple-strand structure.

#### Distinction Between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with

gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

#### SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For

transcription from a transgene in vivo or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to in vitro use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22*<sup>9</sup>, *unc-54*<sup>12</sup>, *fem-1*<sup>14</sup>, and *hlh-1*<sup>15</sup>).

FIGS. 2A–I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-*unc22A*). Panels D–F show progeny of animals injected with ds-*gfpG*. Panels G–I demonstrate specificity. Animals are injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20  $\mu$ m.

FIGS. 3A–D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show in situ hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3*

RNA<sup>20</sup>). Panel C: Embryo from a parent injected with purified *mex-3B* antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to *mex-3B*; no *mex-3* RNA was detected. Scale: each embryo is approximately 50  $\mu$ m in length.

FIG. 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0–6 hours, 2 for 6–15 hours, 3 for 15–27 hours, 4 for 27–41 hours, and 5 for 41–56 hours. The bottom-left diagram shows genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids<sup>8,3</sup>.

FIGS. 5A–C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3) expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole

organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxy-acid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panicle response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may

be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12–16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may be a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphelenchus, Criconemella, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Meloidiogyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and

acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art<sup>32,33,34</sup> (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or otherwise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or

tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology. Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells in vitro or ex vivo and then subsequently placed into an animal to affect therapy, or directly treated by in vivo administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovium, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomasclerosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, diges-

tive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophosphorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxigenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in Index of plant Diseases in the United States (U.S. Dept. of Agriculture Handbook No. 165, 1960); Distribution of Plant-Parasitic Nematode Species in North America (Society of Nematologists, 1985); and Fungi on Plants and Plant Products in the United States (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial

nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones

of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The pesticide of the present invention may serve as an arachnicide, insecticide, nematocide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds,

shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

#### Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene<sup>1,2</sup>. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a variety of studies to manipulate gene expression<sup>3,4</sup>.

Despite the usefulness of RNAi in *C. elegans*, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference<sup>3,4</sup>. The only precedent for such a situation was in plants where the process of cosuppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give

effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference<sup>3,4</sup> had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for unc-22 and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific in vitro promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters in vivo, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

#### Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The unc-22 gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between unc-22 gene activity and the movement phenotypes of animals<sup>3,8</sup>: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. unc-22 encodes an abundant but non-essential myofilament protein<sup>7-9</sup>. unc-22 mRNA is present at several thousand copies per striated muscle cell<sup>3</sup>.

Purified antisense and sense RNAs covering a 742 nt segment of unc-22 had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (FIG. 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (FIG. 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. unc-22 expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands,

were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism<sup>10</sup>. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to unc-22 did not potentiate the ability of unc-22 single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in cis to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded unc-22 segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by unc-22 dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic unc-22 loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (FIG. 1 and Table 1). unc-54 encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction<sup>7,11,12</sup>, fem-1 encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production<sup>13,14</sup>, and hlh-1 encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility<sup>15,16</sup>. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment unc54C, which led to an embryonic and larval arrest phenotype not seen with unc-54 null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes<sup>17</sup>. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The unc54C segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The unc-54 and hlh-1 muscle phenotypes, in particular, are known to result from a large number of defective muscle cells<sup>11,16</sup>. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to gfp produced dramatic decreases in the fraction of fluorescent cells (FIG. 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with gfp inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived

striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13–14 versus 8–9 for embryonic muscles<sup>18,19</sup>). At high concentrations of gfp dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or postembryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (FIG. 3). Here, a mex-3 transcript that is abundant in the gonad and early embryos<sup>20</sup> was targeted, where straightforward in situ hybridization can be performed. No endogenous mex-3 mRNA was observed in animals injected with a dsRNA segment derived from mex-3 (FIG. 3D), but injection of purified mex-3 antisense RNA resulted in animals that retained substantial endogenous mRNA levels (FIG. 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for unc-22, gfp, or lacZ into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient “nicking” of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal’s pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see FIG. 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals with dsRNA.

The *C. elegans* gene unc-22 encodes an abundant muscle filament protein. unc-22 null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from unc-22, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the unc-22 gene. The *C. elegans* fem-1 gene encodes a late component of the sex determi-

nation pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to fem-1, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a gfp transgene were fed bacteria expressing dsRNA corresponding to the gfp reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see FIG. 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from fem-1 and gfp produced no twitching, dsRNAs from unc-22 and fem-1 did not reduce gfp expression, and dsRNAs from gfp and unc-22 did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either gfp or unc-22 caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The unc-22 dsRNA was segment ds-unc22A from FIG. 1. pos-1 and sqt-3 dsRNAs were from the full length cDNA clones. pos-1 encodes an essential maternally provided component required early in embryogenesis. Mutations removing pos-1 activity have an early embryonic arrest characteristic of skn-like mutations<sup>29,30</sup>. Cloning and activity patterns for sqt-3 have been described<sup>31</sup>. *C. elegans* sqt-3 mutants have mutations in the col-1 collagen gene<sup>31</sup>. Phenotypes of affected animals are noted. Incidences of clear phenotypic effects in these experiments were 5–10% for unc-22, 50% for pos-1, and 5% for sqt-3. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that unc-22 dsRNA produced only Unc-22 phenotypes, pos-1 dsRNA produced only Pos-1 phenotypes, and sqt-3 dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806–811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M. K. & Fire, A. Trends in Genetics, 14, 255–258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions<sup>21</sup> for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

#### Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase<sup>6</sup>, followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original “sense” and “antisense” preparations. Nonetheless,



RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68° C. to remove secondary structure, sense+antisense annealing was carried out in injection buffer<sup>27</sup> at 37° C. for 10–30 minutes. Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA in vitro. Non-annealed sense+antisense RNAs for unc22B and gfpG were tested for inhibitory effect and found to be much more active than the individual single strands, but 2–4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for unc22A, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of  $0.5 \times 10^6$  to  $1.0 \times 10^6$  molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

#### Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with gfp<sup>27</sup> and lacZ activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (myo-3 promoter driving mitochondrially targeted GFP), pSAK2 (myo-3 promoter driving a nuclear targeted GFP-LacZ fusion), and a dpy-20 subclone<sup>26</sup> as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the micro-injection needle into the gonadal syncytium of adults and expelling 20–100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic sever-

ity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

#### Additional Description of the Results

FIG. 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: unc-22<sup>9</sup>, unc-54<sup>12</sup>, fem-1<sup>14</sup>, and hlh-1<sup>15</sup>). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., unc22C). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (FIG. 2, panels A–H). These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-unc22A RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (FIG. 2A), adult (FIG. 2B), and adult body wall at high magnification (FIG. 2C).

In contrast, the progeny of animals injected with ds-gfpG RNA are affected (FIGS. 2D–F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (FIG. 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (FIG. 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in FIG. 2F). Inhibition was target specific (FIGS. 2G–I). Animals were injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in FIG. 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (FIG. 2H). Scale bars in FIG. 2 are 20  $\mu$ m.

The effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA was shown by in situ hybridization to embryos (FIG. 3, panels A–D). The 1262 nt mex-3 cDNA clone<sup>20</sup> was divided into two segments, mex-3A and mex3B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. mex-3B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and in situ hybridization (see

Reference 5). The mex-3B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to mex-3A were used to assay distribution of the endogenous mex-3 mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (FIG. 3A). Embryos from uninjected parents showed a normal pattern of endogenous mex-3 RNA (FIG. 3B). The observed pattern of mex-3 RNA was as previously described in Reference 20. Injection of purified mex-3B antisense RNA produced at most a modest effect: the resulting embryos retained mex-3 mRNA, although levels may have been somewhat less than wild type (FIG. 3C). In contrast, no mex-3 RNA was detected in embryos from parents injected with dsRNA corresponding to mex-3 (FIG. 3D). The scale of FIG. 3 is such that each embryo is approximately 50  $\mu$ m in length.

Gene-specific inhibitory activity by unc-22A RNA was measured as a function of RNA structure and concentration (FIG. 4). Purified antisense and sense RNA from unc22A were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (gfpG). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids<sup>8, 3</sup>.

FIGS. 5A–C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (FIG. 5A). A bacterial strain (BL21/DE3)<sup>28</sup> expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (FIG. 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (FIG. 5C). As an alternative

strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either unc-22 or gfp. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

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TABLE 1

Gene and Segment		Effects of sense, antisense, and mixed RNAs on progeny of injected animals.	
		Size Injected RNA	F1 Phenotype
unc-22		unc-22 null mutants: strong twitchers <sup>7,8</sup>	
unc22A <sup>a</sup>	exon 21–22	742 sense	wild type
		antisense	wild type
		sense + antisense	strong twitchers (100%)
unc22B	exon27	1033 sense	wild type
		antisense	wild type

TABLE 1-continued

Effects of sense, antisense, and mixed RNAs on progeny of injected animals.			
Gene and Segment	Size	Injected RNA	F1 Phenotype
unc22C fem-1	exon 21–22 <sup>b</sup>	785 sense + antisense sense + antisense	strong twitchers (100%) strong twitchers (100%) fem-1 null mutants: female (no sperm) <sup>13</sup>
fem1A	exon 10 <sup>c</sup>	531 sense antisense sense + antisense	hermaphrodite (98%) hermaphrodite (>98%) female (72%)
fem1B unc-54	intron 8	556 sense + antisense	hermaphrodite (>98%) unc-54 null mutants: paralyzed <sup>7,11</sup>
unc54A	exon 6	576 sense antisense sense + antisense	wild type (100%) wild type (100%) paralyzed (100%) <sup>d</sup>
unc54B	exon 6	651 sense antisense sense + antisense	wild type (100%) wild type (100%) paralyzed (100%) <sup>d</sup>
unc54C	exon 1–5	1015 sense + antisense	arrested embryos and larvae (100%)
unc54D	promoter	567 sense + antisense	wild type (100%)
unc54E	intron 1	369 sense + antisense	wild type (100%)
unc54F hlh-1	intron 3	386 sense + antisense	wild type (100%) hlh-1 null mutants: lumpy-dumpy larvae <sup>16</sup>
h/h1A	exons 1–6	1033 sense antisense sense + antisense	wild type (<2% lpy-dpy) wild type (<2% lpy-dpy) lpy-dpy larvae (>90%) <sup>e</sup>
hlh1B	exons 1–2	438 sense + antisense	lpy-dpy larvae (>80%) <sup>e</sup>
hlh1C	exons 4–6	299 sense + antisense	lpy-dpy larvae (>80%) <sup>e</sup>
hlh1D myo-3 driven GFP transgenes <sup>f</sup>	intron 1	697 sense + antisense	wild type (<2% lpy-dpy) makes nuclear GFP in body muscle
<u>myo-3::NLS::gfp::lacZ</u>			
gfpG	exons 2–5	730 sense antisense sense + antisense	nuclear GFP-LacZ pattern of parent strain nuclear GFP-LacZ pattern of parent strain nuclear GFP-LacZ absent in 98% of cells
lacZL myo-3::MtLS::gfp	exon 12–14	830 sense + antisense	nuclear GFP-LacZ absent in >95% of cells makes mitochondrial GFP in body muscle
gfpG	exons 2–5	730 sense antisense sense + antisense	mitochondrial GFP pattern of parent strain mitochondrial GFP pattern of parent strain mitochondrial GFP absent in 98% of cells
lacZL	exon 12–14	830 sense + antisense	mitochondrial GFP pattern of parent strain

## Legend of Table 1

Each RNA was injected into 6–10 adult hermaphrodites (0.5–1×10<sup>6</sup> molecules into each gonad arm). After 4–6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20–22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12–24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each unc22A RNA preparation at a series of different concentrations. At the highest dose tested (3.6×10<sup>6</sup> molecules per gonad), the individual sense and antisense unc22A preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-unc22A RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-unc22A RNA produced visible twitching in 30% of progeny.

b: unc22C also carries the intervening intron (43 nt).

c: fem1A also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (laid at 4–24 hours after injection) showed movement defects indistinguishable from those of null mutants in unc-54. A variable fraction of these animals (25–75%) failed to lay eggs (another phenotype of unc-54 null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indi-

cate partial inhibition of unc-54 activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of hlh-1 inhibitory RNA include arrested embryos and partially elongated L1 larvae (the hlh-1 null phenotype) seen in virtually all progeny from injection of ds-hlh1A and about half of the affected animals from ds-hlh1B and ds-hlh1C) and a set of less severe defects (seen with the remainder of the animals from ds-hlh1B and ds-hlh1C). The less severe phenotypes are characteristic of partial loss of function for hlh-1.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of gfp (loss of all fluorescence) and lacZ (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-gfpG caused a loss of GFP in all but 0–3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0–5 additional bodywall muscles and in the eight vulval muscles.

TABLE 2

Effect of injection point on genetic inhibition in injected animals and their progeny			
dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

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TABLE 3

<i>C. elegans</i> can respond in a gene-specific manner to environmental dsRNA.			
Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	<1% female	<1% faint GFP
BL21(DE3)	0% twitch	43% female	<1% faint GFP
[fem-1 dsRNA]			
BL21(DE3)	85% twitch	<1% female	<1% faint GFP
[unc22 dsRNA]			
BL21(DE3)	0% twitch	<1% female	12% faint GFP
[gfp dsRNA]			

TABLE 4

Effects of bathing <i>C. elegans</i> in a solution containing dsRNA.	
dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of unc-22 function)
pos-1	Embryonic arrest (similar to loss of pos-1 function)
sqt-3	Shortened body (Dpy) (similar to partial loss of sqt-3 function)

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-*unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-*unc22A*. Injections of ds-*gfpG* or ds-*lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-*gfpG* and ds-*lacZL* produced no twitching, while injections of ds-*unc22A* produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

We claim:

1. A method to inhibit expression of a target gene in a cell in vitro comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-stranded molecule, and the double-stranded molecule inhibits expression of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the first ribonucleotide sequence comprises at least 25 bases which correspond to the target gene and the second ribonucleotide sequence comprises at least 25 bases which are complementary to the nucleotide sequence of the target gene.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. A method to inhibit expression of a target gene in an invertebrate organism comprising:

(a) providing an invertebrate organism containing a target cell, wherein the target cell contains the target gene and the target cell is susceptible to RNA interference, and the target gene is expressed in the target cell;

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(b) contacting said invertebrate organism with a ribonucleic acid (RNA), wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide sequences are separate complementary strands that hybridize to each other to form the double-stranded molecule; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

13. The method of claim 12 in which the organism is a nematode.

14. The method of claim 13 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

15. The method of claim 12 in which said double-stranded ribonucleic acid structure is at least 25 bases in length and each of the ribonucleic acid strands is able to specifically

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hybridize to a deoxyribonucleic acid strand of the target gene over the at least 25 bases.

16. The method of claim 12 in which the expression of the target gene is inhibited by at least 10%.

17. The method of claim 12 in which the RNA is introduced within a body cavity of the organism and outside the target cell.

18. The method of claim 12 in which the RNA is introduced by extracellular injection into the organism.

19. The method of claim 12 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

20. The method of claim 19 in which the food comprises a genetically-engineered host transcribing the RNA.

21. The method of claim 12 in which at least one strand of the RNA is produced by transcription of an expression construct.

22. The method of claim 21 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 6,506,559 B1  
DATED : January 14, 2003  
INVENTOR(S) : Andrew Fire et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page.

Item [73] Assignee, should read:

-- [73] Assignee: **The Carnegie Institution of Washington**, Washington, DC (US);  
**The University of Massachusetts**, Boston, Massachusetts (US) --

Signed and Sealed this

Sixteenth Day of September, 2003

A handwritten signature in black ink, appearing to read "James E. Rogan", with a long horizontal flourish underneath.

JAMES E. ROGAN  
*Director of the United States Patent and Trademark Office*

# Exhibit 2

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## RNA-Mediated RNA Degradation and Chalcone Synthase A Silencing in Petunia

M. Metzlauff, M. O'Dell,  
P. D. Cluster,\* and R. B. Flavell  
John Innes Centre  
Norwich Research Park  
Colney, Norwich, NR4 7UH  
United Kingdom

### Summary

Transgenic *Petunia* plants with a *chsA* coding sequence under the control of a 35S promoter sometimes lose endogene and transgene chalcone synthase activity and purple flower pigment through posttranscriptional *chsA* RNA degradation. In these plants, shorter poly(A)<sup>+</sup> and poly(A)<sup>-</sup> *chsA* RNAs are found, and a 3' end-specific RNA fragment from the endogene is more resistant to degradation. The termini of this RNA fragment are located in a region of complementarity between the *chsA* 3' coding region and its 3' untranslated region. Equivalent *chsA* RNA fragments remain in the white flower tissue of a nontransgenic *Petunia* variety. We present a model involving cycles of RNA-RNA pairing between complementary sequences followed by endonucleolytic RNA cleavages to describe how RNA degradation is likely to be promoted.

### Introduction

The silencing of transgenes and of endogenous genes homologous to transgenes is a frequently observed phenomenon in plants (for an overview, see Meyer, 1995). The possible mechanisms of silencing may vary among the reported cases (Flavell, 1994; Matzke and Matzke, 1995). Sometimes they result in inhibition of transcription (Meyer and Heidmann, 1994); in other cases they act posttranscriptionally (De Carvalho Niebel et al., 1995; Depicker et al., 1996; Hamilton et al., 1996). The loss of chalcone synthase in *Petunia* hybrida flowers due to the introduction of *chalcone synthase A* (*chsA*) transgenes is apparently not associated with reduced transcription, as demonstrated by run-on transcription tests in isolated nuclei (Van Blokland et al., 1994). It therefore has been assumed to be due to posttranscriptional degradation of *chsA* RNA from both the endogene and the transgene *chsA* genes. This phenomenon has been called cosuppression (Napoli et al., 1990). Chalcone synthase is a key enzyme in anthocyanin biosynthesis, and the *chsA* genes are transcriptionally activated in the epidermal cells of flower petals (Martin, 1993). Loss of *chsA* RNA therefore leads to loss of anthocyanin pigment in petals, and white flower tissue is created instead of purple tissue.

A large number of transgenic *Petunia* plants with variant flower phenotypes have been described that contain

transgenes consisting of *Petunia chsA* cDNA under the control of the cauliflower mosaic virus 35S promoter with two enhancer elements and the 3' untranslated region (UTR) from the nopaline synthase gene of *Agrobacterium tumefaciens* (Napoli et al., 1990; Jorgensen, 1995; Jorgensen and Napoli, 1996; Jorgensen et al., 1996). Many of the transgenic plants have only white flowers, thus displaying complete phenotypic cosuppression. Others display cosuppression in floral sectors, and the white sectors are organized in specific patterns. Recently, Jorgensen et al. (1996) showed that the frequency and patterns of floral cosuppression in such *Petunia* plants are correlated with the number of transgenes and their arrangement in the genome. The patterns of cosuppression are inherited somatically and sometimes through meiosis. However, in other cases epigenetic changes occur in meristems and result in changes in the floral patterns of cosuppression (Jorgensen, 1995; Jorgensen and Napoli, 1996). Despite the extensive studies on the phenotypic expression of cosuppression and knowledge that the process apparently involves *chsA* RNA degradation (Van Blokland et al., 1994), little is understood about the precise changes in RNA levels in transformants displaying different extents of cosuppression and the mechanisms of specific RNA degradations.

In this report we describe changes in *chsA* RNA biology in leaves and flowers in *Petunia* plants that are induced as a consequence of introducing the *chsA* transgenes. From the amounts and structures of the *chsA* RNAs, we conclude that transgenic plants with purple or purple-white flowers can have elevated levels of endogene and transgene poly(A)<sup>+</sup> *chsA* RNAs, while plants with all-white flowers show substantial loss of endogene and transgene poly(A)<sup>+</sup> RNAs. Much of the *chsA* RNA is poly(A)<sup>-</sup>, which we conclude is a product of a specific cleavage in the mRNA. A *chsA* endogene 3' end-specific RNA fragment appears to be the last endogene RNA fragment to be degraded in tissue showing severe *chsA* cosuppression. An identical remaining *chsA* 3' end fragment can be observed in the white flower tissue of plants, as it can in the nontransgenic *Petunia* variety Red Star, which carries purple-white patterned flowers (Mol et al., 1983); this observation indicates that the transgene RNA present in transgenic lines acts only as a trigger for the induction of an existing *chsA* RNA-specific, posttranscriptional control mechanism. We present a model based on RNA-RNA base pairing, when poly(A)<sup>-</sup> or aberrant RNAs are locally elevated, and involving specific endonucleolytic RNA cleavages to explain how *chsA* RNA degradation is likely to be promoted to cause phenotypic cosuppression.

### Results

#### Amounts of *chsA* RNA Differ between Leaf and Flower Tissues and among Transformants with Different Floral Phenotypes

To determine the relative levels of *chsA* RNA in leaf and flower tissues of wild-type and selected transgenic

\* Present address: Plant Breeding Laboratory, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan.

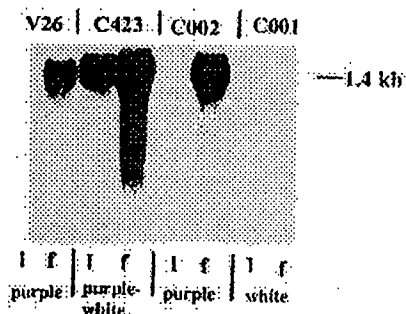


Figure 1. Northern RNA Analysis of Leaf and Flower Tissues of Wild-Type and Selected Transgenic Petunia Plants

Ten micrograms of total RNA was separated on a 1% agarose-20% formaldehyde gel, transferred onto a nylon filter, and hybridized with a  $^{32}$ P-*chsA*-cDNA probe. The size of the *chsA* transcript and the flower phenotypes are indicated. l, leaves; f, flowers.

Petunia plants, we performed Northern RNA analyses (Figure 1) and RNA dot-blot analyses (data not shown). Both the Northern and the dot-blot analyses proved the high induction of *chsA* RNA levels in wild-type flowers relative to leaves (Figure 1, V26) and the strong reduction of *chsA* RNA in white flower tissue (Figure 1, C001). In this latter tissue only traces of *chsA* RNA could be detected by both analyses, whereas hybridization of the filter with ribosomal DNA proved that approximately equal amounts of total RNA were transferred onto the filter. The drastic reduction in the level of *chsA* RNA in white flower tissue shown in our Northern and dot-blot analyses is consistent with data published earlier for Petunia flower tissue with cosuppressed *chsA* genes (Napoli et al., 1990; Van der Krol et al., 1990; Van Blokland et al., 1994).

The total RNA analyses yielded two other findings. First, in purple flower tissue of the two transgenic lines C423 and C002, the level of *chsA* RNA was higher than in wild-type purple flower tissue. The observed elevation ranged between 20- to 40-fold, as estimated from a series of dot-blot analyses with dilutions of total RNA down to 0.015  $\mu$ g. Second, the level of *chsA* RNA in leaf tissue of the unstable, purple-white flowering transgenic line C423 was much higher (>100-fold) than the expected very low levels in wild-type leaves and in leaves of stable purple (C002) or stable white (C001) flowering transgenic lines.

To investigate whether the presence of specific *chsA* RNA species in leaf and flower tissue correlates with specific phenotypes, we carried out endogene- and transgene-specific reverse transcriptase polymerase chain reaction (RT-PCR) analyses on total leaf and flower RNA preparations. By using specific oligonucleotides (normally 20-mers) as primers for cDNA synthesis and PCR amplifications, we differentiated between endogene and transgene *chsA* transcripts and between total *chsA* RNA and polyadenylated *chsA* RNA. The cDNA reactions were carried out on equal aliquots of RNA. This approach allowed quantitative comparisons to be made within and between genotypes. To aid the quantitative interpretation of PCR products, relatively few amplification cycles (normally 18) were carried out,

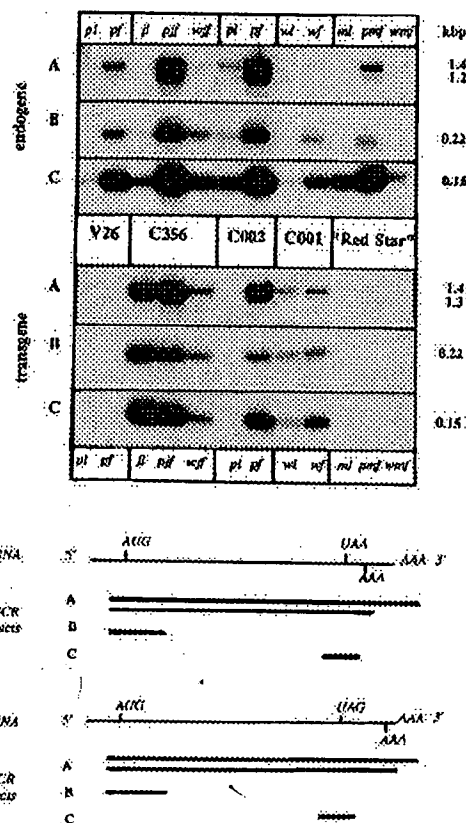


Figure 2. RT-PCR Performed on Total RNA Isolated from Leaf and Flower Tissues of Nontransgenic and Selected Transgenic Petunia Plants

Endogene and transgene *chsA* RNA-specific cDNAs were amplified by 18 cycles of PCR. The resulting PCR products were separated on either 1.5% (A) or 2.5% (B and C) agarose gels, transferred onto nylon membrane, and hybridized with a *chsA*-cDNA probe. The sizes of the RT-PCR products are indicated. V26 is the purple flowering wild-type line, Red Star a nontransgenic line with purple-white patterned flowers, C356 a transgenic line with purple-white patterned flowers, C002 a transgenic line with fully purple flowers, and C001 a transgenic line with all-white flowers. The type of tissue from which the total RNAs were isolated are labeled as follows: pl, leaf tissue of plants with fully purple flowers; pf, purple flower tissue; jl, leaf tissue of plants with purple-white patterned flowers; pjf or wjf, purple or white sectors, respectively, of purple-white patterned flowers; wl, leaf tissue of plants with fully white flowers; wf, white flower tissue; ml, leaf tissue of nontransgenic plants with purple-white patterned flowers; pmf or wmf, purple or white sectors, respectively, of nontransgenic purple-white flowers.

followed by hybridization with a *chsA* cDNA probe. Where RT-PCR products could not be detected by hybridization, a high number of PCR amplification cycles (normally 35) was performed, followed by hybridization, to investigate whether very low levels of *chsA* RNAs were present.

Figure 2 summarizes RT-PCR data for plants selected as representatives of three phenotypic groups: plants with stable phenotypes of all-purple flowers (C002, which is an epigenetic variant of C001; Napoli et al., 1990); plants with all-white flowers (C001); and plants with purple-white patterned flowers (C356). We com-



pared the RT-PCR patterns for the transgenic lines with RT-PCR patterns obtained for total RNA isolated from leaf and flower tissue of wild-type V26 plants and from flowers similar to those of the *Petunia* variety Red Star. We decided to include this variety in our analyses because its flowers display purple and white sectors like those of the unstable purple-white flowering transgenic lines. To assay poly(A)<sup>+</sup> RNA we performed 3' rapid amplification of cDNA ends (RACE) amplifications (Figure 2A; Frohman et al., 1988) using an oligo(dT)-adaptor primer. To display full-length *chsA* poly(A)<sup>+</sup> RNA and to differentiate between endogene and transgene poly(A)<sup>+</sup> cDNA, the adaptor oligonucleotide was used in combination with the 5' UTR-specific oligonucleotides G995 and G996, respectively, for the 18 PCR cycles. A single RT-PCR product of 1.4 kb was expected for full-length poly(A)<sup>+</sup> RNAs, from both the endogene and the transgene. To detect any nonpolyadenylated *chsA* RNA in addition to poly(A)<sup>+</sup> RNA displayed by 3' RACE, we primed other cDNA syntheses using identical RNA preparations with 5' end- and 3' end-specific oligonucleotides (Figures 2B and 2C, respectively). For the 5' end-specific cDNA syntheses we used the oligonucleotide G2350, which is complementary to nucleotides 1360 to 1381 within the *chsA* coding region (numbering according to sequence file X14591 of the EMBL/GenBank/DBJ databases). To distinguish between endogene and transgene 5' ends, we amplified aliquots of the resulting cDNAs using the oligonucleotide G2350 in combination with the same oligonucleotides G995 or G996 that we used for the full-length poly(A)<sup>+</sup> RNA RT-PCR amplifications. The expected size of the resulting RT-PCR products was 220 bp in both cases. The 3' end-specific cDNA syntheses were primed either with oligonucleotide G1607, which is complementary to the first 20 nucleotides of the endogene 3' UTR, or G1607T, which is complementary to the first 20 nucleotides of the transgene 3' UTR. Both 3' end-specific cDNAs were PCR amplified with either G1607 or G1607T in combination with oligonucleotide G3280, which is specific for positions 3611–3634 within the *chsA* coding region. The expected size for both RT-PCR products was 150 bp.

Our analyses showed that *chsA* poly(A)<sup>+</sup> RNA can be displayed by RT-PCR at high levels in all types of purple flower tissue (Figure 2, pf, pjf, and pmf). In the case of wild-type and Red Star flower tissue, only endogene-specific RT-PCR products were obtained, whereas both types of *chsA* poly(A)<sup>+</sup> RNA RT-PCR products, endogene and transgene, were present in transgenic purple flower tissue. However, in the flower tissues of these transgenic genotypes the level of endogene *chsA* poly(A)<sup>+</sup> RNA was clearly elevated in comparison to levels found in wild-type and Red Star-type flower tissue. Thus, here the coexpression of both types of *chsA* genes appears to deregulate the control of endogene *chsA* poly(A)<sup>+</sup> RNA levels. Furthermore, when the level of *chsA* poly(A)<sup>+</sup> RNA is elevated, shorter poly(A)<sup>+</sup> RNA RT-PCR products with sizes of 1.2 kb for the endogene RNA and 1.3 kb for the transgene RNA become abundant. The shorter endogene poly(A)<sup>+</sup> RNA product can be observed as a minor component in RT-PCR analyses performed with RNA from wild-type and Red Star-type flower tissue. We assume therefore that the 1.2 kb RT-PCR product reflects an enhanced *chsA* RNA-specific

modification occurring as a consequence of *chsA* RNA accumulation. For white flower tissue of the transgenic lines (Figure 2, wjf, wf, and wmf) almost no endogene and only low levels of transgene *chsA* poly(A)<sup>+</sup> RNA RT-PCR products were found. This result reflects the strong reduction of *chsA* poly(A)<sup>+</sup> RNA in all-white flower tissue and is consistent with the loss of pigmentation in this tissue.

Our RT-PCR analyses also proved that the level of endogene *chsA* poly(A)<sup>+</sup> RNA in leaves is very low. However, in plants of the stable purple flowering transgenic line C002, the level of endogene *chsA* poly(A)<sup>+</sup> RNA was clearly elevated in leaves but no transgene *chsA* poly(A)<sup>+</sup> RNA could be detected. In contrast, in leaf tissues of the unstable purple-white flowering line C356 and the stable white flowering line C001, relatively high amounts of transgene *chsA* poly(A)<sup>+</sup> RNA product could be displayed by RT-PCR. The amounts were especially high in plants of the unstable purple-white flowering line. From this we conclude that transgene *chsA* poly(A)<sup>+</sup> RNA is the main component of the high levels of *chsA* RNA in leaf tissue of unstable purple-white flowering plants observed earlier in our Northern analyses (Figure 1). The leaves of all of the white flowering transformants that we assayed had low levels of transgene RNA. This supports the conclusion that loss of *chsA* RNA also occurs in the stems and leaves of plants that display cosuppression of *chsA* in flowers. The state of cosuppression in flowers is therefore likely to be determined by the properties of somatic cells long before flower formation.

#### Most of the Surviving *chsA* Transcripts in Flower Tissue Showing Severe Cosuppression Are Not Polyadenylated, and a Portion of the 3' End of the Endogene *chsA* RNA Survives Degradation Preferentially

The data we obtained for the 5' and 3' end-specific RT-PCR analyses of the endogene and transgene *chsA* total RNA confirmed in general our observations of elevated or reduced levels of *chsA* poly(A)<sup>+</sup> RNAs in defined leaf and flower tissues of transgenic plants, but they also revealed other features. In white flower segments of C356, the ratio of poly(A)<sup>+</sup> 1.4 kb endogene RNA fragments to the 0.22 kb 5' end fragments and 0.15 kb 3' end fragments was low, relative to that in C356 purple flowers. This indicates that most of the *chsA* endogene RNA fragments are poly(A)<sup>+</sup>, and the 1.4 kb fragments appear to be more sensitive to the degradation processes than other shorter *chsA* RNA fragments.

The white flower tissue of C001 also had reduced levels of the 1.4 kb poly(A)<sup>+</sup> RNA relative to C002, and here the 0.22 kb 5' fragments were also relatively reduced. In the white flower tissue of Red Star, only 3' end fragments could be detected. There was variation in the extent of total *chsA* RNA reduction, endogene and transgene, and varying residual levels of full-length, 5', and 3' fragments, but 3' fragments always seemed to be the last to disappear from the complement of RNAs. This situation also was observed in leaf tissue of Red Star (Figures 2A–2C). Here the levels of the 3' end fragments were much higher than in V26.

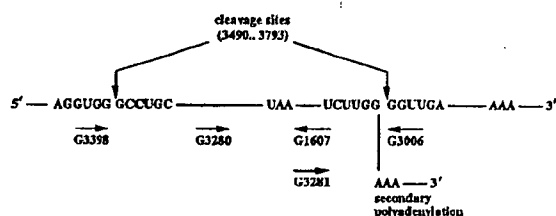


Figure 3. Sequence of Specific RNA Termini Located at the Endogenous *chsA* 3' End

The sequences surrounding identified termini at the endogenous *chsA* 3' end are shown. The numbers of the first 5' nucleotide (3490) and the last 3' nucleotide (3793) of the RNA fragment that are more resistant to degradation *in vivo* are given according to the numbering in sequence file X14591 of the EMBL/GenBank/DBJ databases. The stop codon UAA and the secondary poly(A) site are indicated, as are the numbers and location of oligonucleotides used in 3' end-specific RT-PCR amplifications.

#### The Endogenous *chsA* RNA 3' End Fragments That Resist Degradation Carry Specific 5' and 3' Termini Located in a Region of RNA-RNA Complementarity

The discovery that most *chsA* endogenous RNA fragments in white flower tissue (and leaves) are not full-length poly(A)<sup>+</sup> and that 3' end fragments appear to survive more than 5' end fragments suggested, first, that there may be specific cleavage sites within the *chsA* endogenous RNA, and second, that the 3' end fragments may adopt a structure that is more resistant to RNases. We therefore identified the 3' and 5' ends of the 3' fragments that are most resistant to RNases in white flower tissue. To do this we performed linear PCR reactions toward the *chsA* RNA 3' end and primer extension reactions toward the 5' end from known positions and assayed the sizes of the predominant products on gels. As template for the linear PCR amplifications we used poly(A)<sup>+</sup> cDNA and labeled primer G3281, which is specific for the first 20 nucleotides of the endogenous *chsA* 3' UTR (Figure 3). In the primer extension reactions performed on total RNA we used oligonucleotide G1607, which is complementary to the first 20 nucleotides of the endogenous *chsA* 3' UTR. The positions of the location of the 5' and 3' termini derived from the sizes of major bands of primer extension and linear PCR reactions in white flower tissue are indicated in Figure 3. To verify precisely the terminal bases, the regions surrounding the termination PCR products were sequenced, and the products of the sequencing reactions and primer extensions and PCR reactions were compared on a phosphonoacetic acid (PAA) sequencing gel. The terminal sequences obtained ("cleavage sites" in Figure 3) have been compared with other published RNA truncations points and endonucleolytic cleavage sites. Consistent with the other sequences the last nucleotide is G and the termini are within AU-rich regions (Table 1). The identified termini imply that the remaining *chsA* fragments are 304 bases long. The 3' termination of the 304-base *chsA* RNA fragment deduced from sequencing and of the shorter (1.2 kb) *chsA* poly(A)<sup>+</sup> RNA observed earlier in our 3' RACE experiments (Figure 2A) are identical (data not shown). From this we conclude that identical site-specific endonucleolytic cleavages occur in the formation of both classes of RNA.

To verify that most *chsA* endogenous RNA is poly(A)<sup>-</sup> due to cleavage at the specific 3' cleavage site and that some of the RNA molecules are also cleaved at the 5' site to leave 304-base RNAs, a further series of experiments was conducted on RNA preparations from different tissues from various genotypes. Those from Red Star are shown in Figure 4. Using total RNA isolated from leaf, purple flower, and white flower tissue of Red Star, we primed the three cDNA syntheses with oligonucleotide G3006, which is complementary to the first 20 nucleotides downstream of the defined 3' UTR cleavage site (Figure 3). In subsequent PCR amplifications of aliquots of the cDNAs this oligonucleotide was combined either with oligonucleotide G3398, which is specific for the first 20 nucleotides upstream of the observed 5' termination at the *chsA* 3' end (Figure 4A), or with oligonucleotide G3280, which is located inside the remaining *chsA* RNA 3' fragment (Figure 4C). In both cases a major RT-PCR product was obtained only for the purple flower tissue RNA and not for the leaf tissue or white flower tissue RNAs. In Figure 4, lanes B and D, the cDNA synthesis was primed with oligonucleotide G1607, which is complementary to the first 20 nucleotides of the endogenous *chsA* 3' UTR and is located inside the remaining *chsA* RNA 3' fragment. For PCR amplifications this oligonucleotide was again combined with G3398 located outside or G3280 located inside the remaining RNA fragment. The quantity of PCR products was higher for the purple flower RNA than for the leaf or white flower RNAs, reflecting the higher levels of *chsA* RNA in flowers. The amount of PCR products obtained using a primer downstream of the 3' cleavage site (Figures 4A and 4C) were much lower than those obtained using primers upstream of the 3' cleavage site. This finding confirms that a high proportion of all *chsA* endogenous RNAs, in all tissues, terminate at this site and are poly(A)<sup>-</sup>. The ratios of fragments amplified with the 5' primer outside versus inside the 5' cleavage site (Figures 4B and 4D), as judged by hybridization ratios in the autoradiograph, were similar in the leaf and purple flower tissue, indicating that many but not necessarily all of the RNAs do not have 5' ends at the 5' cleavage site. The equivalent ratio of amplified fragments (Figures 4B and 4D) from white flower tissue is, however, much lower than for leaf or purple flower tissue. In equivalent experiments using RNAs from some other white flower genotypes (data not shown), considerably lower ratios were seen. We therefore conclude from screening many genotypes that under conditions of severe cosuppression—that is, when most of the *chsA* endogenous RNA is degraded—a high proportion of the remaining *chsA* endogenous RNA consists of 304-base 3' end fragments.

We investigated the secondary structure of the 3' end of the *chsA* RNA by computer-based folding analyses in the program FOLD RNA (Zuker and Stiegler, 1981). It showed extensive secondary structures (Figure 5). We noted that in all of the putative secondary structures generated, the termini of the 304-base *chsA* RNA fragment are located within a 43-base paired segment showing 80% complementarity between a *chsA* 3' coding region segment and the *chsA* 3' UTR (Figure 5). Both fragment termini are also located close to bases of intra-strand loops.

Table 1. Truncation Points in the 3' Region of *chsA* RNA (A) in Comparison with Two Published Cases of mRNA Truncations (B)

A Endogene <i>chsA</i> 3' UTR <i>chsA</i> 3' coding region Transgene <i>chsA</i> 3' nos UTR	5'-UUGUUUCUUGG[GGUUG-3' 5'-UUGCUCUCCAG[UGGGCCU-3' 5'-UGAUUAUCAU[AUAAUUU-3' 5'-GAAACCAUG[GJUGA-3' 5'-GGUCUACAG[AACA-3' 5'-AUAAA[GIAACAAGGUGUUU-3'
B Truncation points in the open reading frame of the tobacco etch virus coat protein RNA (Goodwin et al., 1996) Endonucleolytic cleavage site in the 3' UTR of human transferrin receptor mRNA (Binder et al., 1994)	

# The Complementarity between a Segment at the 3' End of the *chsA* Coding Region and the *chsA* 3' UTR Allows In Vitro Formation of RNA Duplexes That Are Not Degraded by a Double-Stranded RNA-Specific Endonuclease

From sequencing data of the 5' and 3' termination points at the *chsA* 3' end and the putative structural features surrounding these terminations we postulated that double-stranded RNA (dsRNA)-specific endonucleases

may be involved in the formation of the 304-base RNA fragment by *chsA* RNA-specific degradation processes. To test this hypothesis, we carried out the in vitro experiment documented in Figure 6. Both complementary regions of the *chsA* endogene 3' end, the segment of complementarity within the 3' coding region (positions 3467–3532 in sequence file X14591 of the EMBL/GenBank/DBJ databases), and the complete 3' UTR (positions 3743–3970), were separately integrated into the in vitro transcription vector pSPT18 (Boehringer Mannheim), from which the recombinant plasmids pCC9 and pET5 resulted. The inserts were transcribed in vitro by using either T7 or SP6 RNA polymerase. In Figure 6, lanes A and B, the <sup>32</sup>P-labeled sense transcripts of the *chsA* 3' UTR were annealed in vitro with a 10-fold excess of unlabeled 3' UTR antisense transcripts. An aliquot of this annealing reaction was incubated with RNase III at 30°C for 20 min (Figure 6B). RNase III is a dsRNA-specific *E. coli* enzyme (Li et al., 1993) and was a gift from Dr. A. Nicholson (Wayne State University). In Figure 6, lanes C and D, the <sup>32</sup>P-labeled sense transcripts of the segment of complementarity at the *chsA* 3' end of the coding region were annealed with a 10-fold excess of unlabeled sense transcripts of the *chsA* 3' UTR. An aliquot of this annealing reaction was also incubated with RNase III. All four reactions were separated on a 5% nondenaturing PAA gel and exposed to X-ray film. In both annealing reactions without subsequent RNase III incubation (Figure 6, lanes A and C), bands with lower mobility than the <sup>32</sup>P-labeled in vitro transcripts could be observed. From this we conclude that in both reactions complex RNA structures have formed. The smear of radioactive products observed is presumably due to the diversity of complex dsRNA structures. Aliquots of both annealing reactions were incubated with RNase III. All of the complex RNA structures were degraded in the case of the self-annealed 3' UTR transcripts (Figure 6, lane B), but where the *chsA* coding and 3' UTR transcripts were mixed, the smear of products disappeared but a product remained with higher mobility than the major products present before RNase III treatment (Figure 6, lane D). We therefore conclude that the complementary coding and 3' UTR RNA sequences can interact to form a structure that may have a cleavage site for RNase III but most of which is resistant to RNase III. The structure is therefore different from that formed when completely complementary RNA sequences are reannealed.

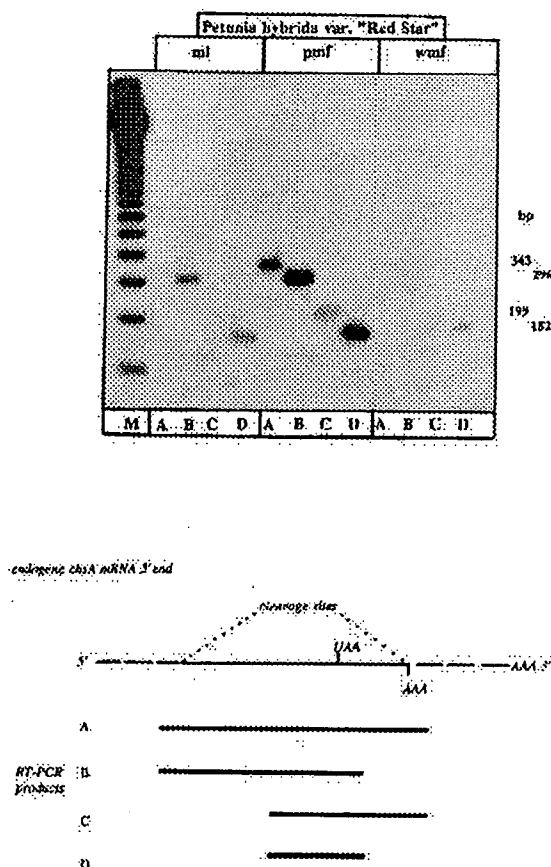


Figure 4. RT-PCR Patterns Obtained for the Endogene *chsA* 3' End  
RT-PCR amplifications were carried out on total RNA isolated from leaf tissue (ml) and purple (pmf) or white (wmf) flower tissue of the nontransgenic *Petunia* line Red Star. The sizes of the resulting RT-PCR products are indicated. The following oligonucleotide combinations were used for the RT-PCR amplifications: A, G3006/G3398; B, G1607/G3398; C, G3006/G3280; and D, G1607/G3280. For the location of these oligonucleotides see also Figure 3. M, 100 bp marker.

## Discussion

From our studies on three groups of transgenic plants—(1) those that produce only fully purple flowers and rarely

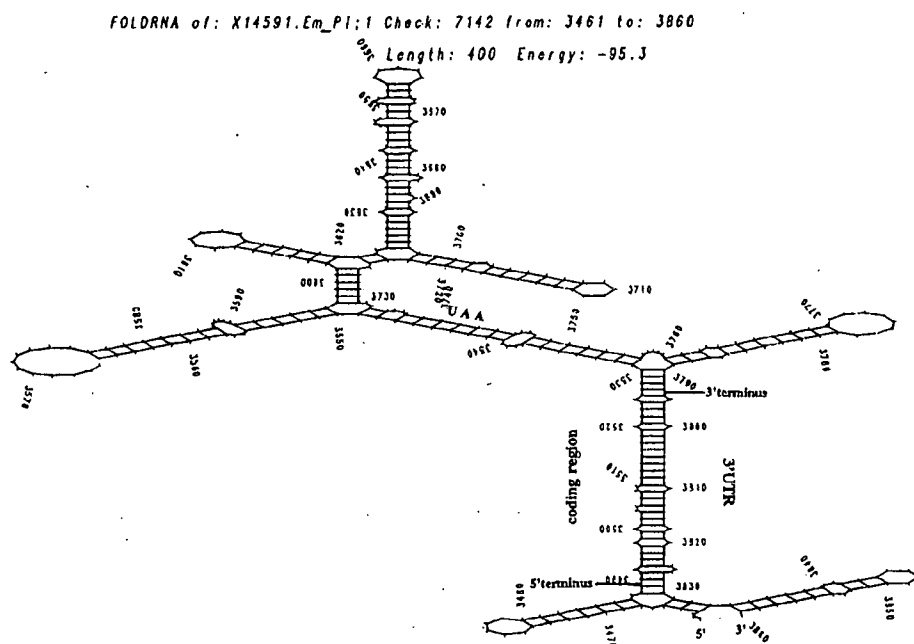


Figure 5. Computer-Based RNA Folding Analysis for the Endogene *chsA* 3' End

The putative secondary structure of the last 400 nucleotides of the endogene *chsA* RNA 3' end was obtained by using the program FOLDRNA (Zuker and Stiegler, 1981). The locations of the UAA stop codon and the 5' and 3' termini of the 304-base *chsA* RNA fragment that resists degradation are indicated.

show floral cosuppression, except sometimes in anthers; (2) those that are prone to cosuppression and regularly show characteristic white floral sectors and also produce somatic side shoots with fully white flowers; and (3) those that routinely produce all-white flowers and show full cosuppression—we can infer a series of steps that lead to cosuppression.

#### Transgenes Provoke a Range of Aberrations in Chalcone Synthase RNAs

When active transgenes do not promote floral cosuppression, as in group 1 plants, higher levels of poly(A)<sup>+</sup> endogene RNA accumulate in floral tissue and their own poly(A)<sup>+</sup> transcripts accumulate to high levels, too (Figure 2). The higher levels of endogene poly(A)<sup>+</sup> RNA imply that the transgenes either stimulate endogene transcription or reduce its degradation. Chalcone synthase RNA is known to be turned over relatively rapidly, and there is evidence that chalcone synthase levels are regulated in part posttranscriptionally (for a review see Martin, 1993). Thus, we favor the view that high concentrations of *chsA* RNA molecules can "titrate out" or inhibit some step in the *chsA* turnover process in flowers, thus reducing overall *chsA* RNA degradation rates. The high levels of endogene and transgene RNAs in these purple-flowered plants, which do not display cosuppression, suggest that high levels of *chsA* RNA alone are insufficient to promote cosuppression.

In the purple-white-flowered plants of group 2, which are prone to floral cosuppression, the *chsA* RNA levels can be typical of the group 1 stable purple types (Figure

2) or can have lower levels (unpublished data) that suggest that the RNA degradation system characteristic of cosuppression is already operating, during plant development, to some extent.

In group 3 plants, those showing full floral cosuppression, no or only low levels of full-length endogene and transgene *chsA* poly(A)<sup>+</sup> RNA survive. However, poly(A)<sup>-</sup> RNA fragments remain in white flower tissue, and when *chsA* RNA degradation is most extensive, 304-base sequences from the 3' end of the RNA remain.

These results suggest that transformants suffer a continuum of aberrations, ranging from inhibition of *chsA* RNA degradation processes to extensive *chsA* degradation. The analyses of the *chsA* RNA surviving in the various transformants suggest that an early step in RNA degradation may be endonucleolytic cleavages at defined positions in the endogene and transgene 3' UTRs. The abundance of the 1.2 kb endogene RNA suggests that this may be the result of the most common initial cleavage site. A putative poly(A) signal (5'-AATTGAA-3') is present 33–27 nucleotides upstream of this 3' UTR cleavage site, which may account for why some RNAs cleaved at this site are polyadenylated. This explanation implies that the cleavage occurs in the nucleus, where polyadenylation also occurs. We note that this putative preferred RNA cleavage site follows a G residue, which is also the case for other RNA endonucleolytic cleavage sites (Table 1B) (Binder et al., 1994; Goodwin et al., 1996; Scheper et al., 1996). The boundaries of the short endogene RNA fragments surviving preferentially in white-flowered plants showing severe cosuppression

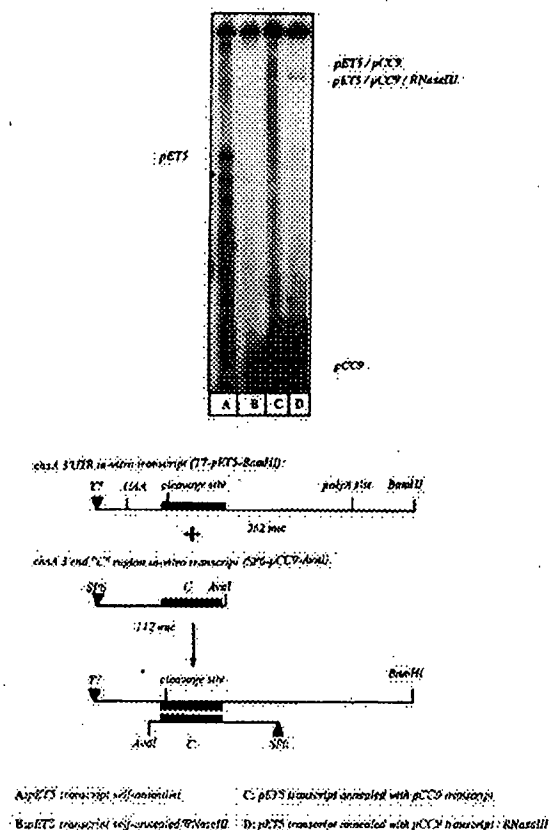


Figure 6. In Vitro Transcription, Annealing, and Endonucleolytic Cleavage of Complementary *chsA* RNA Regions

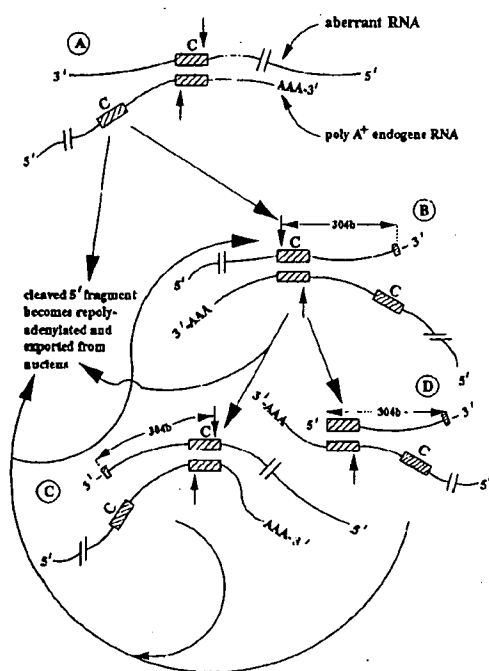
<sup>32</sup>P-labeled in vitro transcripts were either self-annealed or cross-annealed and were separated on a 5% nondenaturing PAA gel. (A) A <sup>32</sup>P-labeled sense transcript of the complete *chsA* 3' UTR was self-annealed with a 10-fold excess of its unlabeled complementary transcript. (B) An equivalent aliquot of this self-annealed RNA was incubated with dsRNA-specific RNaseIII. (C) A <sup>32</sup>P-labeled sense transcript of the complementary region located at the 3' end of the *chsA* coding region was annealed with a 10-fold excess of unlabeled sense transcript of the complete *chsA* 3' UTR. (D) An equivalent aliquot of the RNA mix used in (C) was treated with RNaseIII.

imply that a second cleavage occurs upstream within the *chsA* coding region (Figure 3). The preferential survival of the RNA products of these two endonucleolytic cleavages is especially noteworthy. The RNAs presumably adopt secondary structures that are more resistant to RNases relative to other segments of the *chsA* RNAs. We have shown that some RNA structures formed in vitro between complementary sequences in this RNA segment can resist RNase III degradation unlike other RNA duplexes (Figure 6). The same RNA cleavages occur and the same RNA fragments survive degradation in white flower tissue of plants similar to the nontransgenic variety Red Star (Mol et al., 1983). This finding strongly supports the hypothesis that the transgene *chsA* RNA present in transgenic lines acts only as a trigger for the induction of an existing *chsA* RNA-specific, posttranscriptional control mechanism.

### A Model for Cosuppression Involving Cycles of RNA Pairing and Endonucleolytic Cleavage

If *chsA* RNAs are susceptible to a series of cleavages, how can the presence of transgenes stimulate these cleavage processes? Jorgensen et al. (1996) have shown that cosuppression increases with more copies of the gene. Here we show that reversion from white to stable purple flowers in the epigenetic formation of C002 from C001 is associated with a large reduction in the levels of transgene RNA in leaves. It is unlikely that high endogene expression is required to maintain the state of cosuppression in transgenic plants because cosuppression of transgene RNAs is observed in tissues during plant development when endogene expression is low. Endogene expression occurs only in the L1 epidermal cells, but transgene RNA levels show suppression also in flower petal L2 layers. We therefore conclude that transgene RNA is sufficient to maintain the state of cosuppression, even though cosuppression might be enhanced by the presence of endogene RNA, for example in epidermal cells of purple-white patterned flowers. However, we concluded earlier that high levels of *chsA* alone are insufficient to promote cosuppression. We therefore suggest that aberrant poly(A)<sup>−</sup> *chsA* RNA is the active inducer of cosuppression.

Much long poly(A)<sup>−</sup> *chsA* RNA accumulates in plants possessing active transgenes and in plants similar to the variety Red Star. This conclusion is based on the relatively low levels of full-length poly(A)<sup>+</sup> RNA relative to 5' and 3' sections of RNA in white versus purple tissues (Figure 2). We do not know what induces the production of poly(A)<sup>−</sup> RNA fragments but suspect that it may be the localized accumulation of high levels of RNA. The presumed preferential sites of endonucleolytic cleavage of endogene RNA lie within 43-base paired segments of the coding regions and 3' UTR sequences that are 80% complementary. These cleavages in endogene RNA might therefore result from the recognition of structures formed by intermolecular pairing between the coding sequence on aberrant fragments of RNA and the complementary 3' UTR sequence of the mRNA. Cleavage of this paired RNA, as shown in Figure 7, would produce the 1.2 kb form of endogene mRNA observed in Figure 2 and a shorter poly(A)<sup>−</sup> aberrant RNA. The 1.2 kb endogene RNA has a poly(A) addition sequence and could explain why some of these molecules are repolyadenylated. On the assumption that the 1.2 kb endogene RNAs are formed in this way, we predict that the pairing and cleavages take place in the nucleus; otherwise, repolyadenylation could not take place. Poly(A)<sup>−</sup> endogene products from this pairing-cleavage reaction presumably would not be actively transported out of the nucleus because of lack of a poly(A) tail and therefore could base pair with the 3' UTR complementary sequences of other full-length endogene mRNAs to produce, following endonucleolytic cleavages, more of the 1.2 kb endogene RNAs and the 304-base fragments observed (Figure 7). We therefore propose that loss of *chsA* RNA in floral cosuppression occurs by means of pairing-cleavage cycles between poly(A)<sup>−</sup> endogene or transgene RNA molecules that fail to progress out of the nucleus efficiently and nuclear full-length mRNA



**Figure 7. A Cyclic Model of RNA Degradation Based on Complementary RNA Pairing and Endonucleolytic Cleavage**

The process is initiated by the local accumulation of aberrant *chsA* RNAs, which can be any *chsA* RNA, from endogene or transgene, whose "normal" export from the nucleus and translation are inefficient or prevented because of structural aberrations. The aberrant RNA base pairs with the complementary sequence in the 3' UTR of an endogene RNA (A). Endonucleolytic cleavages occur at specific sites (short line pairs) to generate two truncated molecules. The truncated endogene RNA can be repolyadenylated and exported from the nucleus to form the observed shorter poly(A)<sup>+</sup> endogene RNA. Alternatively, it can pair with the 3' UTR sequence of another *chsA* endogene RNA (B). Specific endonucleolytic cleavages of this duplex lead to the observed 304-base RNA and a long *chsA* RNA lacking its 3' end. This latter RNA can be repolyadenylated and exported from the nucleus, or it can pair with the 3' UTR sequence of another *chsA* endogene RNA (C), as in (B). The 304 bp product from (B) can also pair with another endogene RNA (D) to produce another long RNA lacking its 3' end. Thus the products of one pairing-cleavage event are potential substrates for two others. The same cycle can also take place between RNAs in the cytoplasm.

products, as a result of the sequence complementarity between the coding and 3' UTR sectors. We have shown (Figure 6) that RNA-RNA pairing in vitro between these sectors can lead to structures that are cleaved by *E. coli* dsRNA-specific RNase III but are not degraded extensively like RNA-RNA duplexes between completely complementary RNA sequences. However, it remains unknown whether RNase III-like enzymes act in vivo as an endonucleolytic component of the observed *chsA* RNA-specific RNA degradation system.

Once the process of *chsA* RNA-specific degradation is initiated, it could eliminate all of the endogene full-length *chsA* RNA rapidly because the products of each reaction are substrates for another round of reactions, as shown in Figure 7. The proposed "autoregulatory" degradative cycle is thus attractive to explain (1) how the observed relatively large proportions of poly(A)<sup>-</sup>

*chsA* RNA are generated; (2) how phenotypic switches from purple to white tissue occur rapidly; and (3), if the short RNA molecules survive through the cell cycle, how the state of cosuppression is somatically inherited. The attractions of an autoregulatory system to explain gene silencing observations have been described by Meins and Kunz (1994). In addition, the 1.4 kb endogene RNAs with two sites capable of undergoing complementary pairing with transgene RNA or with poly(A)<sup>-</sup> cleavage products would be expected to undergo degradation more rapidly than 1.2 kb endogene and transgene 1.4 kb mRNAs, which have only one site, as is frequently observed (Figure 2 and unpublished data). Although we suggest that pairing-cleavage cycles occur in the nucleus, it is also likely that they occur in the cytoplasm, if the conformation of the RNAs in the mRNPs allows intermolecular interactions and the relevant RNases are present.

The origins of cosuppression in this model are the observed aberrant *chsA* poly(A)<sup>-</sup> RNA molecules. They occur in nontransgenic situations, but their concentration is enhanced in transgenic lines or in conditions of high levels of *chsA* endogene RNA. In this regard it is noteworthy that low levels of the 1.2 kb poly(A)<sup>+</sup> endogene RNA molecules are seen in wild-type *Petunia* and in plants similar to the variety Red Star.

The proposed RNA degradation scheme is based on the existence of complementary sequences in *chsA* mRNA that are located at the mRNA 3' end. Other examples exist where interactions between the 3' UTR and other mRNA sequences are involved in the regulation of RNA cleavage by guiding RNA cleavage (Wightman et al., 1993). However, similar complementary sequences that promote RNA-RNA pairing may exist in other regions of a mRNA. Such complementarity may have been selected as a component of an RNA turnover control system by intra- or intermolecular RNA pairing.

Why do some transgenic plants with active *chsA* transgenes have stable purple flowers? The reason could be that the transgenes are localized in nuclear sectors that give rise to less poly(A)<sup>-</sup> *chsA* RNA, that transcription rates are insufficiently high to accumulate enough *chsA* RNA in the nucleus, or that the mRNAs of endogenes and transgenes are in different regions of the nucleus. Epigenetic switches in cosuppression may therefore be due to changes in nuclear organization or transgene expression, as noted for C001 and C002 here. They occur most frequently in meristems and affect L1 and L2 layers simultaneously, possibly because meristems are where resetting of nuclear organization and transgene or endogene chromatin occurs preferentially. The state of cosuppression may also be activated by conditions that activate *chsA* endogenes. A boost to the level of endogene RNA would enhance the pool of molecules able to interact and drive the cycle to a new steady state of RNA degradation. It is therefore relevant that cosuppression can be influenced by external conditions also known to influence *chsA* expression (Jorgensen and Napoli, 1996).

The differences in floral patterns observed for different lines displaying *chsA* cosuppression may reflect the differential expression of transgenes in response to special gradients of transgene transcription factors in sectors of

the meristem (Jorgensen, 1995; Jorgensen et al., 1996). However, from the model proposed here they may also reflect the patterns of endogene expression, the locations of transgene and endogene *chsA* RNAs in the nucleus, the pattern of accumulation of poly(A)<sup>-</sup> *chsA* RNAs, or conceivably the patterns of movement of small cleavage products of *chsA* RNAs between cells in the meristem.

Jorgensen et al. (1996) have concluded from a large study of transgenic plants that single copies of transgenes stimulate cosuppression only during flower development (i.e., when endogene RNA is activated), whereas multiple copies of transgenes, and especially inverted repeat copies, enhance the probability of more extensive cosuppression and, in particular, cosuppression in leaves and stems. These structures might promote aberrant transgene RNAs. Our model implies that transgene expression is essential for transgenes to stimulate the occurrence of cosuppression. However, Van Blokland et al. (1994) have reported that promoterless transgenes can stimulate cosuppression. We cannot explain this observation, but it may be due to transcription from another promoter at some point in development to initiate the RNA pairing-cleavage cycle or due to generation of an abnormal RNA in low quantities that has a very high potential of being poly(A)<sup>-</sup> and thereby initiating the endogene RNA pairing-cleavage cycle. If, as we predict here, RNA-RNA pairing exists in the nucleus to drive the phenomenon of cosuppression, RNA-DNA pairing might also occur to cause epigenetic changes to the genes. Elsewhere we will present data on such epigenetic changes that correlate with cosuppression.

Finally, our data and the model described here are also consistent with recent reports about cases of posttranscriptional gene silencing in *Neurospora crassa* (Cogoni et al., 1996) and in virus-resistant transgenic plants (Baulcombe and English, 1996; English et al., 1996; Goodwin et al., 1996). Dougherty et al. (1994) and Smith et al. (1994) have offered an explanation for the means by which cytoplasmic viral RNA replication is inhibited in transgenic plants possessing low levels of transgene mRNA homologous to the viral RNA. Our model, derived from the data on chalcone synthase, has many similarities to theirs; however, having defined an RNA degradation product carrying a complementary sequence, we favor the conclusion that cytoplasmic RNA pairing-cleavage cycles are also the basis of transgene-promoted virus resistance. The regions of complementarity within RNA molecules would be expected to differ from case to case and may not be restricted to 3' end positions. There has been considerable debate on whether antisense RNA is involved in posttranscriptional sense-gene silencing (Grierson et al., 1991; Mol et al., 1991; Flavell, 1994). In the model proposed here, complementary RNA is involved, but it is inherent in the sense transcript.

#### Experimental Procedures

##### Transgenic Plants

The transgenic *Petunia hybrida* plants used in our experiments were kindly provided by Dr. John Bedbrook (DNA Plant Technology Corporation, Oakland, California) and Dr. Richard Jorgensen (Depart-

ment of Environmental Horticulture, University of California, Davis). The original transformants described by Napoli et al. (1990) and Jorgensen et al. (1996) were backcrossed by us with wild-type plants V26 to give C001, C002 (from CHS38), C423 (from CHS244), and C356 (from CHS223). A distinct description of the transgenic *Petunia* lines including the transgene copy numbers can be found in Jorgensen et al. (1996). In our comparative analyses we used the non-transgenic *Petunia* F1 Razzle Dazzle (Suttons Seeds Ltd., UK) with a flower pattern similar to that of the variety Red Star (Mol et al., 1983). Plants of this variety show white sectors in purple flower tissue like that of purple-white patterned transgenic plants.

##### *chsA* Transgene Construction and Plant Transformations

The *chsA* transgene construction and plant transformations were carried out at DNA Plant Technology Corporation and at the Department of Environmental Horticulture, University of California, Davis, as described in Napoli et al. (1990) and Jorgensen et al. (1996).

##### Northern and Dot-Blot RNA Analyses

Northern and dot-blot RNA analyses were carried out by following the Stratagene instruction manual. Total RNA was isolated as described by Napoli et al. (1990) from freshly harvested leaves and flowers immediately frozen in liquid nitrogen. The age of the plants used in our analyses varied between 6 weeks to 3 months. For Northern analyses, 10 µg of total RNA was separated on 1% agarose-20% formaldehyde gels. All hybridization probes were labeled by random priming using [ $\alpha$ -<sup>32</sup>P]deoxycytosine triphosphate ([ $\alpha$ -<sup>32</sup>P]dCTP) (Amersham).

##### RT-PCR

RT-PCR analyses were performed following the RACE protocol (Frohman et al., 1988) with some modifications. One microgram of total RNA dissolved in sterile water was mixed with 2 µl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 25 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40), 2 µl of 5 mM deoxynucleotide triphosphate mix (prepared by mixing equal volumes of 20 mM deoxynucleotides, Boehringer Mannheim), 2 µl of 50 µM RACE oligo dT-adaptor (B26-B25) or 2 µl of 20 µM primer complementary to sense RNA, and water to a final volume of 19 µl. The mixture was heated at 65°C for 5 min and rapidly cooled on ice. After the addition of 1 µl of RT (Moloney murine leukemia virus [M-MLV] RT, GIBCOBRL), the cDNA synthesis was carried out at 52°C for 30 min. The RT was subsequently inhibited by heating the samples at 99°C for 1 min. Aliquots of the resulting cDNA were used for PCR with final primer concentrations of 0.5 µM and 0.5 µl Taq-polymerase (Boehringer Mannheim). The PCR reactions were carried out for 18 or 35 cycles, respectively (30 s at 94°C, 30 s at 60°C, 1 min at 72°C) and a final extension at 72°C for 10 min. Ten microliters of the total reaction volumes were used for electrophoresis on 1.5%-2.5% agarose gels in Tris-borate-EDTA buffer. The DNA was transferred with 20× saline-sodium citrate onto nylon membrane and hybridized with <sup>32</sup>P-labeled probes.

##### Linear PCR

For linear PCR reactions, cDNA aliquots were amplified in a total reaction volume of 20 µl using 50 nM of a single <sup>32</sup>P-end-labeled oligonucleotide for 18 PCR cycles (20 s at 94°C, 30 s at 60°C, 30 s at 72°C) and a final extension at 72°C for 10 min. Two microliters of each reaction was separated on 6% PAA-urea sequencing gels.

##### PCR Sequencing of DNA Fragments

Dideoxy-PCR sequencing of DNA fragments isolated from agarose gels was carried out according to the protocol of Murray (1989). Fifty nanograms of DNA template and 50 nM of a <sup>32</sup>P-end-labeled primer were used for 18 cycles of amplification (performed as for linear PCR) without a final extension at 72°C. Two microliters of the resulting sequencing reaction were separated on 6% PAA-urea sequencing gels.

##### In Vitro Transcription, Transcript Annealing, and RNase III Cleavage

PCR fragments for the region of complementarity within the *chsA* 3' end coding region and for the complete *chsA* 3' UTR were separately ligated with SmaI-cut and T-tailed pSPT18 vector DNA (Boehringer Mannheim) from which the recombinant plasmids pCC9 and pET5

resulted. The *in vitro* transcription reactions were carried out according to the instructions of the supplier by using either T7 or SP6 RNA polymerase and 50  $\mu$ Ci [ $\alpha$ - $^{32}$ P]CTP (Amersham) or unlabeled CTP. After phenol extraction and ethanol precipitation, the resulting *in vitro* transcripts were mixed in a ratio of 1 to 10 (labeled to unlabeled). The mixed transcripts were heated at 90°C for 10 min followed by an incubation at 30°C for 1 hr. Aliquots of the annealing reactions were incubated with 1 Kunitz unit of *E. coli* RNase III at 30°C for 20 min in a buffer containing 60 mM KCl, 10 mM MgCl<sub>2</sub>, and 5 mM Tris-HCl (pH 7.5). Products of the *in vitro* reactions were then separated on 5% nondenaturing PAA gels.

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## The Silence of Genes in Transgenic Plants

MAIKE STAM, JOSEPH N. M. MOL and JAN M. KOOTER\*

*Department of Molecular Genetics, Institute for Molecular Biological Sciences, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands*

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In genetically modified plants, the introduced transgenes are sometimes not expressed. They can be silenced. Transgenes can also cause the silencing of endogenous plant genes if they are sufficiently homologous, a phenomenon known as co-suppression. Silencing occurs transcriptionally and post-transcriptionally but silencing of endogenous genes seems predominantly post-transcriptional. If viral transgenes are introduced and silenced, the post-transcriptional process also prevents homologous RNA viruses from accumulating; this is a means of generating virus-resistant plants. A major goal of current research is to dissect the mechanism(s) of these sequence-homology-dependent gene silencing phenomena. Various factors seem to play a role, including DNA methylation, transgene copy number and the repetitiveness of the transgene insert, transgene expression level, possible production of aberrant RNAs, and ectopic DNA–DNA interactions. The causal relationship between these factors and the link between transcriptional and post-transcriptional silencing is not always clear. In this review we discuss various observations associated with gene silencing and attempt to relate them.

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### INTRODUCTION

Analysis of the large collection of genetically modified plants generated in recent years has expanded our knowledge of physiological processes and gene regulation mechanisms tremendously. However, transgenes do not always behave as expected. This has revealed the existence of hitherto unknown cellular processes. There is considerable variation in the expression of transgenes in individual transformants which is not due to differences in copy number. Thus, gene activity is not exclusively determined by the strength of the promoter which controls transcription; epigenetic effects also influence expression levels. This sometimes leads to gene inactivation either by blocking transcription or by inhibiting mRNA accumulation. Until now, gene silencing has been a confusing field of research. Most of us stumbled upon this phenomenon fortuitously by analysing transgenic plants. The mechanisms by which silencing is achieved are still poorly understood. Despite the different silencing systems being examined, several cases of gene silencing have features in common which gives us insight into the factors involved.

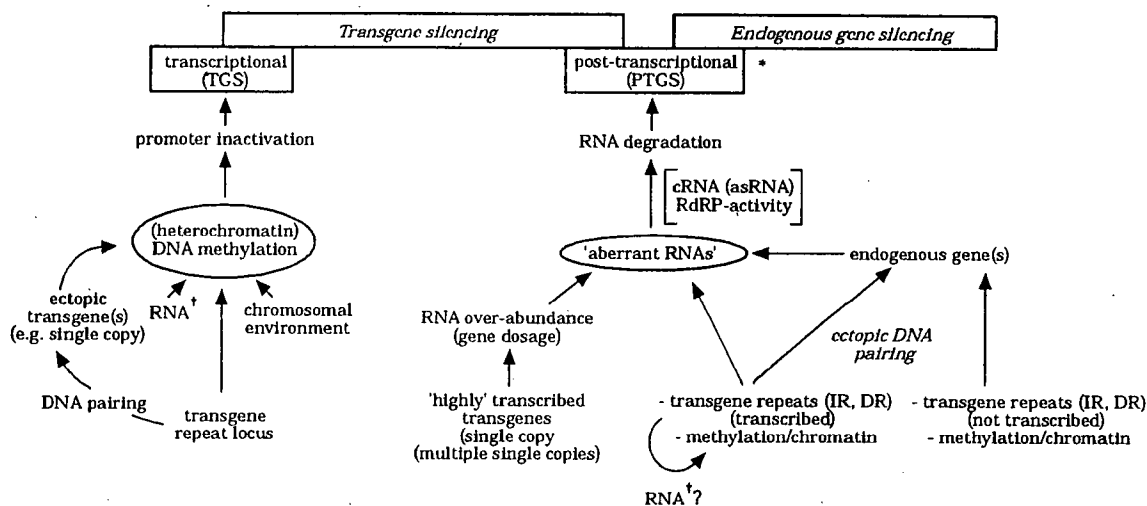
Gene silencing also occurs in untransformed plants where it reduces expression of endogenous genes. A number of mutations in various plant species appear to result from epigenetic gene silencing. For example, paramutation in maize (Brink, 1973) and tomato (Hageman, 1993) probably involve gene–gene interactions. Although paramutation was known long before the discovery of transgene-mediated silencing, it is only recently that the underlying molecular mechanisms have become apparent. Several features re-

semble those associated with transgene-mediated silencing. Because of space limitations, we will only discuss the silencing of transgenes and endogenous genes in transgenic plants. A detailed description of paramutation and related phenomena in untransformed plants can be found in other reviews (Matzke and Matzke, 1993; Patterson and Chandler, 1995) and some recent articles (Das and Messing, 1994; Hollick *et al.*, 1995; Patterson *et al.*, 1995; Ronchi, Petroni and Tonelli, 1995). Various aspects of transgene-mediated silencing discussed here can also be found in other reviews (Finnegan and McElroy, 1994; Flavell, 1994; Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe and English, 1996; Meyer, 1996) and books (Paszukowski, 1994; Meyer, 1995; Grierson, Lycett and Tucker, 1996).

### SILENCING OF TRANSGENES

Various possibilities have been raised to explain variation in transgene expression levels among transformants that is independent of copy number. All these imply that integrated transgenes cannot be regarded as independent transcription units. Transgenes, often as part of the *Agrobacterium tumefaciens* T-DNA, integrate at different chromosomal locations. If they become inserted into euchromatin, in a transcriptionally active region (Koncz *et al.*, 1989; Herman *et al.*, 1990; Kertbundit *et al.*, 1991), expression may be influenced by regulatory sequences of nearby host genes. If they insert in or near repetitive DNA or heterochromatin, they can be inactivated (Pröls and Meyer, 1992). Another important factor associated with gene silencing is the number of transgenes per integration site. The T-DNA transfer system can insert two or more T-DNAs at the same chromosomal site. These T-DNAs can be arranged 'head-

\* For correspondence.



al., 1993). This has been observed with a maize *Al* gene in petunia whereby only the inserted DNA was methylated (Meyer and Heidmann, 1994). This *de novo* methylation of foreign DNA is thought to be a cellular defence response against the potential harmful activity of this DNA (Doerfler, 1995). How DNA is recognized as being 'foreign' is unknown, but it might be related to a different adenine-thymine (AT) content relative to that of the flanking DNA (Salinas et al., 1988; Matassi et al., 1989; Meyer and Heidmann, 1994; Elomaa et al., 1995).

**Repeat-induced DNA methylation.** Transgenes of T-DNAs which are inserted as a DR or an IR have a tendency to become inactivated. This is frequently associated with DNA methylation (Hobbs, Kpodar and Delong, 1990; Kilby et al., 1992; Assaad et al., 1993; Hobbs et al., 1993; Vaucheret, 1993; Matzke et al., 1994; Stam and Kooter, unpubl. res.). The trigger of this *de novo* methylation is unknown but is probably a response to the repetitive nature of the locus. But how? In the case of IRs it might be the ability to create a cruciform which is a good substrate for DNA methyltransferases (Laayoun and Smith, 1995). In general, the more copies a locus contains, the stronger the inactivation (Matzke et al., 1994). Assaad et al. (1993) were able to follow recombination derivatives of a transgene locus that contained repetitive sequences. Predominantly, the multi-gene recombinants showed silencing. These cases of repeat-induced gene silencing (RIGS) resemble phenomena in other eukaryotes, such as repeat-induced point mutation (RIP) in *Neurospora crassa* (Singer, Marcotte and Selker, 1995; Singer and Selker, 1995) and methylation-induced pre-meiotically (MIP) in *Ascomobolus immersus* (Rhounim, Rossignol and Faugeron, 1992; Rossignol and Faugeron, 1995). In these fungi, inactivation of tandemly duplicated genes is very efficient and there is evidence to suggest that it is mediated by DNA-DNA pairing (Selker and Garrett, 1988).

**DNA-DNA interactions and gene silencing in trans.** A transgene locus that is silenced can silence homologous transgenes at ectopic loci (Matzke et al., 1989; Vaucheret, 1993; Matzke et al., 1994). When the silencing locus is methylated, the target locus also becomes methylated. In the case of homologous promoters this transfer of methylation can lead to transcriptional inactivation. This has been described in detail for a potent silencer locus, the 271 transgene locus, which contains antisense nitrite reductase genes driven by a strong CaMV-35S promoter (Vaucheret, 1993; Elmayan and Vaucheret, 1996; Park et al., 1996). This silencing locus contains multiple copies and appears very efficient in silencing homologous sequences elsewhere in the genome which are then methylated. When and how this interaction occurs, and how it leads to methylation is unknown, but some of the possibilities are discussed by Matzke and Matzke (1995).

Methylation of the target genes is erased after crossing out the silencing locus. This does not happen immediately but occurs gradually in successive generations (Matzke and Matzke, 1991) or even during a plant's lifetime (Vaucheret, 1994). Thus, to maintain the fully inactive state, the silencing locus needs to be present permanently, suggesting regular cross-talk between the homologous sequences. Cross-talk

between homologous transgene sequences might be enhanced in homozygous plants. An inactive single copy transgene is even able to inactivate an active allele *in trans*, probably by an allelic interaction (Meyer et al., 1993). This and other results (see below) indicate that even with single copy insert transformants, which usually give the least problems concerning stable transgene expression, one occasionally encounters gene inactivation in plants homozygous for the transgene. Potentially, this may cause problems in breeding programmes where inbred lines are used. It occurs quite often that transgenes in hemi- and homozygous plants behave differently. In many cases silencing is only observed or is severely enhanced in homozygous plants (e.g. De Carvalho et al., 1992; Hart et al., 1992; Dehio and Schell, 1994; Brandle et al., 1995; Vaucheret et al., 1995; Elmayan and Vaucheret, 1996; Howie et al., 1996). It is interesting to note that silencing also occurs in haploid plants that were generated from anthers from non-silenced hemizygous plants (De Carvalho et al., 1992; Elmayan and Vaucheret, 1996). These results indicate that an allelic interaction between transgene loci is not necessary in homozygous plants for the induction of silencing, merely the gene dosage per genome is important.

#### POST-TRANSCRIPTIONAL SILENCING OF TRANSGENES AND HOST GENES

Transcriptional silencing as a result of promoter inactivation by DNA methylation and/or heterochromatinization is conceptually straightforward, even though many of the underlying molecular events need to be worked out. It seems more complicated when gene silencing results from a post-transcriptional process (Fig. 1, right-hand side). In this case, promoters are active and the genes transcribed, but mRNA fails to accumulate. The transgene-induced PTGS mechanism affects expression of the transgenes and of endogenous genes with which they share a considerable degree of sequence identity. The latter case is also known as co-suppression because the endogenous genes and the transgenes are silenced. Co-suppression was first observed with genes involved in flower pigmentation (Napoli, Lemieux and Jorgensen, 1990; Van der Krol et al., 1990) and in tomato fruit ripening (Smith et al., 1990). Silencing of the chalcone synthase (*chs*) genes in petunia flowers occurs post-transcriptionally (Van Blokland et al., 1994; Metzlaff, O'Dell, and Flavell, 1996). Using homologous sense transgenes, the expression of many endogenous genes has been suppressed. This approach is now frequently used to study gene function as inactivation of endogenous genes can result in mutant phenotypes (Fig. 2).

#### Characteristics of PTGS

The PTGS mechanism appears to act on any RNA that is homologous to the transgene that has activated it, thus from transgenes, endogenous genes and from transiently expressed genes (Hobbs et al., 1993; English et al., 1996). Also, viral RNAs are attacked when transgenes are used that contain viral sequences (De Haan et al., 1992; Lindbo et al., 1993; Mueller et al., 1995). The same holds for

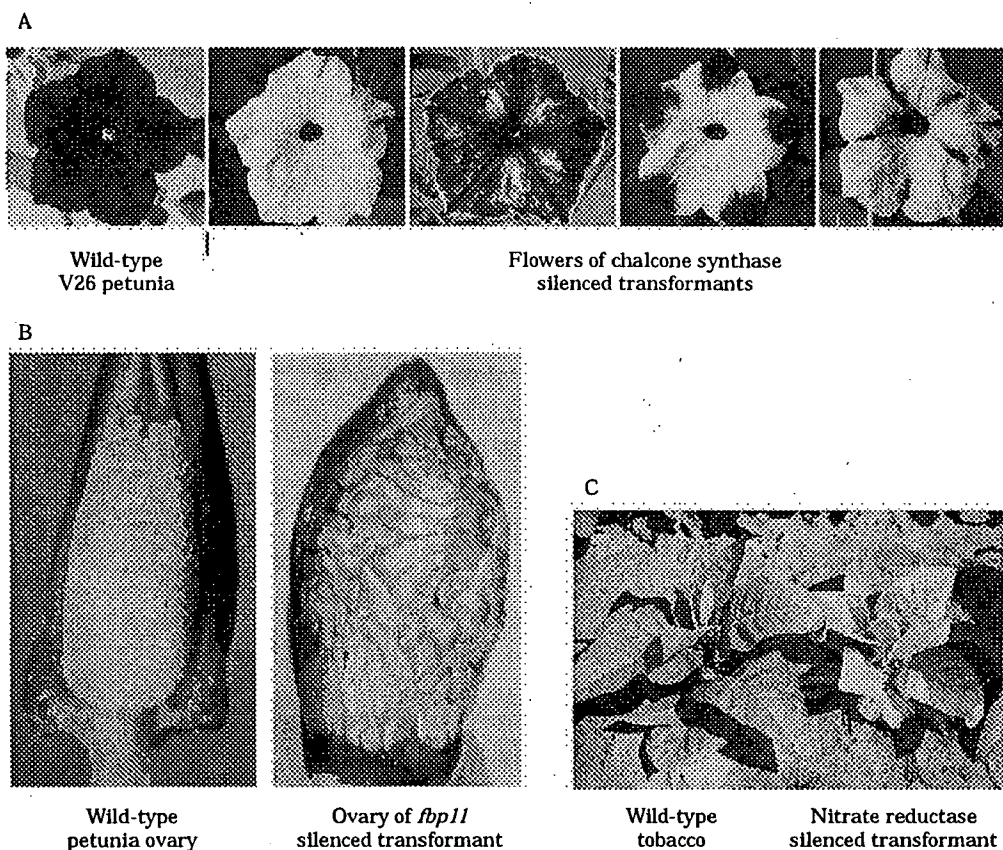


FIG. 2. Phenotypic alterations caused by transgene-induced silencing of endogenous genes. A, Silencing of the anthocyanin biosynthesis gene chalcone synthase (*chs*) in petunia corollas by *chs* sense transgenes. The degree and extent of silencing varies among independent transformants resulting in various pigmentation patterns (Van Blokland *et al.*, 1994; Stam and Kooter, unpubl. res.). On the left is a wild-type V26 flower. The cause of the patterned silencing is unknown. B, Silencing of *fbp11*, a MADS box gene, in petunia transformants containing CaMV-35S driven sense *fbp11* transgenes. This results in aberrant ovule development. On the left is a wild-type ovary; on the right that of the *spaghetti* mutant (Photograph kindly provided by Gerco Angenent; For details, see Angenent *et al.*, 1995). C, Silencing of nitrate reductase expression in tobacco by CaMV-35S promoter driven sense nitrate reductase transgenes which results in chlorotic plants (right). (Photograph kindly provided by Hervé Vaucheret. Reprinted by permission from Molecular & General Genetics. Copyright 1994, Springer Verlag; For details, see Dorlhac de Borne *et al.*, 1994).

engineered chimaeric viruses which contain non-viral sequences. Transformants carrying silenced *uidA*, *nptII* transgenes, or polygalacturonase trans- and endogenous genes, do not accumulate chimaeric viruses that carry sequences of these genes (English *et al.*, 1996). The 'foreign' sequence in the viral RNA acts as a target for the degradation machinery. Interestingly, in the case of *uidA*, only viruses which carried the 3' region of the *uidA* coding region did not accumulate. Thus not every sequence can act as a target for RNA degradation (see below).

The proportion of transformants in which transgenes and/or homologous host genes are silenced varies, but can be very high (Elmayan and Vaucheret, 1996; Jorgensen *et al.*, 1996). The reasons for this are not known exactly, but they seem to be related to transgene expression levels and the organization of the T-DNA inserts. Moreover, silencing does not always occur in every cell of a particular tissue. In the case of anthocyanin gene silencing, this is clearly visible by the various flower coloration patterns (Van der Krol *et al.*, 1990; Van Blokland *et al.*, 1994; Jorgensen, 1995;

Jorgensen *et al.*, 1996) (Fig. 2A). In some systems the PTGS is developmentally regulated. Silencing of *uidA* (Elmayan and Vaucheret, 1996), chitinase (Kunz *et al.*, 1996) and of  $\beta$ -1,3-glucanase transgenes in tobacco (De Carvalho Niebel *et al.*, 1995), does not occur in young seedlings but starts between 2 and 6 weeks after germination, depending on the expression level of the transgenes. A similar meiotically reversible silencing was observed with *rolB* transgenes in *Arabidopsis* (Dehio and Schell, 1994). Taken together, the PTGS mechanism(s) must not only account for the enhanced turnover of specific transcripts but also for the developmental and spatial control of this process.

#### Possible PTGS mechanisms

The hallmark of PTGS is the reduced accumulation of specific transcripts. Whether the reduction results from RNA instability, from activation of a sequence-specific RNA degradation process, or a combination of both, is unknown. The right-hand side of Fig. 1 gives an overview of

several possibilities in an attempt to relate different PTGS features. These include a role for transgene expression level, for the structure and methylation of a multicopy locus, and whether or not the nucleus contains an endogenous homologue. The latter is important because the mRNA elimination process may be activated by means of an interaction of the transgene locus with the endogenous gene(s) (Van Blokland *et al.*, 1994) which might only be possible with particular transgene loci.

**Role of transgene expression level.** An attractive model explaining PTGS invokes an effect of highly expressed transgenes. In this model, the transgenes produce so much RNA that the level exceeds a critical threshold thereby triggering a mechanism that specifically removes all homologous RNAs irrespective of their source. This RNA threshold model gained support from observations whereby PTGS was correlated with highly expressed transgenes conferring viral resistance (Lindbo *et al.*, 1993; Smith *et al.*, 1994; Goodwin *et al.*, 1996). Plants containing highly transcribed transgenes did not accumulate the transgenic mRNAs and were also resistant to infection by an homologous RNA virus (Smith *et al.*, 1994; Goodwin *et al.*, 1996). Plants in which the transgenes were transcribed at lower levels accumulated transgenic RNA normally, but were also sensitive to a viral infection. Sometimes a virus infection is needed to induce the PTGS mechanism and resistance. This result is in line with reaching the hypothetical RNA threshold level (Lindbo *et al.*, 1993). The threshold model may also explain PTGS of highly expressed *uidA* transgenes in tobacco (Elmayan and Vaucheret, 1996) and of single copy *chs* transgenes in petunia (Jorgensen *et al.*, 1996). Also studies by Metzlaff *et al.* (1996) of *chs* co-suppression in petunia transformants suggest that silencing requires active transgenes.

Not all cases of PTGS are associated with highly expressed transgenes. For example, the degree of viral resistance is not always directly related to viral transgene expression (Mueller *et al.*, 1995; English *et al.*, 1996; see below and reviews on strategies and mechanisms of viral resistance by Pang, Slightom and Gonsalves, 1993; Baulcombe, 1994, and Dougherty and Parks, 1995). Also silencing of the *chs* genes in petunia is not always associated with highly transcribed *chs* transgenes (Van Blokland *et al.*, 1994). This suggests that the absolute level of normal mRNAs is not important. It might be more crucial to have sufficient amounts of an RNA that is qualitatively distinct from the regular mRNA. These so-called aberrant RNAs are thought to be required for activating the PTGS mechanism (Dougherty and Parks, 1995; Metzlaff *et al.*, 1996).

**PTGS and methylated transgenes.** PTGS is frequently associated with methylated transgenes (Hobbs *et al.*, 1990, 1993; Ingelbrecht *et al.*, 1994; Smith *et al.*, 1994; English *et al.*, 1996). The PTGSed *nptII* genes described by Ingelbrecht *et al.* (1994) were methylated up- and downstream of the coding region. Also Smith *et al.* (1994) noticed that the viral transgenes of the virus-resistant plants were methylated more than those of the sensitive plants. Despite this methylation, the promoters were active and the genes transcribed, but whether the transcripts were normal is unknown. In *Ascochola immersus*, transcription of meth-

ylated genes gives rise to truncated transcripts (Barry, Faugeron and Rossignol, 1993). Although this was suggested to result from premature termination of transcription, a specific degradation of the RNA part that is transcribed from the methylated DNA region cannot be ruled out. Indeed Ingelbrecht *et al.* (1994) showed that post-transcriptionally silenced *nptII* genes in tobacco were transcribed beyond methylated restriction sites at the 3' end of the gene.

Studies by English *et al.* (1996) provided more direct evidence for a role of DNA methylation in PTGS. The PTGSed *uidA* genes in the tobacco plants described by Hobbs *et al.* (1990, 1993) were more methylated near the 3' end of the gene which included the poly-adenylation region. Importantly, this 3' region was also needed to prevent infection by the PVX-*uidA* chimeric viruses, as if this part of the RNA is the target for the degradation of RNA. Thus, there seems to be a link between DNA-methylation and the RNA-based silencing mechanism (English *et al.*, 1996).

Although in some cases transgene PTGS is associated with methylation, there is no evidence that the post-transcriptionally silenced endogenous genes are methylated (Hart *et al.*, 1992; Stam and Kooter, unpubl. res.), despite the fact that the transgenes contain coding regions which are potentially able to induce methylation of a target locus. Where it has been examined, the promoters of post-transcriptionally silenced endogenous genes are normally active and the genes are transcribed at wild-type levels (Van Blokland *et al.*, 1994; De Carvalho Niebel *et al.*, 1995; Kunz *et al.*, 1996). However, there is one example where transcription of an endogenous gene, *cab140* in *Arabidopsis*, is decreased (five-fold) due to the presence of a *cab140* promoter-driven transgene (Brusslan *et al.*, 1993; Brusslan and Tobin, 1995). This reduced transcription is probably the result of an epigenetic change of the promoter caused by the homology between the promoters of the transgene and the endogenous genes. The molecular basis of the silencing is not yet known but methylation does not seem to be involved (Brusslan *et al.*, 1993).

**RNA-directed DNA methylation (RdDM).** There is one study that provides evidence for a role of RNA in *de novo* methylation of the homologous DNA (Wassenegger *et al.*, 1994). In tobacco transformants it was found that transgenes composed of three or four tandemly arranged viroid cDNAs in sense or antisense orientation and driven by the CaMV-35S promoter were methylated. It was argued that this methylation was caused by the viroid RNAs. However, it cannot be ruled out that methylation of the three and four-copy transgene constructs was due to the repetitive nature of the transgenes (see above). The demonstration that a two-copy and a monomeric viroid cDNA construct, which were unmethylated, became methylated after the plant was infected with viroid RNA and replication had taken place, indicate that the viroid RNA is responsible for the methylation of the homologous transgene DNA rather than the transgene structure or the integration site. How this occurs is unknown but it is conceivable that DNA-RNA hybrids are formed (Wassenegger *et al.*, 1994). These structures might be good substrates of DNA-methyltransferases, like cruciform structures in DNA. Whether this

intriguing RdDM mechanism is just a response to certain nucleic acid interactions or a genuine regulatory mechanism to control gene expression by epigenetic changes remains to be resolved.

Although viroid RNA induces methylation of viroid transgenes which may spread into the promoter region and cause transcriptional silencing, it does not result in a detectable post-transcriptional silencing (and as a result viroid resistance) as is observed for the plus-RNA viruses (PVX, PVY). It is not known why, but one possibility is that the viroid RNAs cannot be reached for degradation as they reside in the nucleus (Harders *et al.*, 1989). At present it is not known if DNA methylation induced by viroid RNA is an exception or that other (abundant) RNAs can do the same. This interesting possibility was raised to explain some of the silencing phenomena involving methylated transgenes (Wassenegger *et al.*, 1994).

**Elimination of homologous transcripts.** If the PTGS mechanism is induced according to the RNA threshold model it implies that cells are able to measure levels of specific RNAs. If this activity functions in normal cells monitoring RNA levels from endogenous genes, one would expect that it also establishes and maintains a certain steady-state RNA level in transgenic cells. This is not the case: the vast majority of homologous RNAs are degraded. Either such an RNA-control activity does not exist in normal cells or it is modified by the products of the transgenes, perhaps as a sort of defence mechanism against the possible harmful effects of these 'foreign' DNAs.

We can only speculate about the nature of this RNA degradation activity. Dougherty's group postulated a role for complementary RNAs (cRNA) synthesized by the plant-encoded RNA-dependent RNA polymerase (RdRP) in the cytoplasm (Lindbo *et al.*, 1993; Dougherty and Parks, 1995). RdRP from tomato was recently characterized (Schiebel *et al.*, 1993a) and it was shown that *in vitro*, this enzyme synthesizes small RNAs from RNA templates (Schiebel *et al.*, 1993b). It is hypothesized that *in vivo* these cRNAs hybridize to complementary mRNAs which are then degraded by double-stranded RNA specific RNases. The appeal of the RdRP-cRNA model is that it explains the strong sequence specificity of PTGS.

In the case of *chs* co-suppression in petunia, Metzlaiff *et al.*, (1996) detected considerable amounts of RNA degradation intermediates derived from the 3' half of the *chs* mRNA. Also smaller 3' end products were detected and the sequence of these smaller RNAs revealed that they were derived from transcripts of which a small segment was removed by cryptic splicing. This unusual splicing event and the finding that unspliced *chs* transcripts in *chs*-silenced tissues accumulate (Van Blokland *et al.*, 1996) suggest that the processing of primary transcripts is to some extent impaired. The response to the presence of these unusual transcripts is unknown but one may speculate that they can act as template for the RdRP.

**PTGS of endogenous genes and the presence of specific transgene loci.** Van Blokland *et al.* (1994) observed silencing of endogenous *chs* genes in petunia with promoterless *chs* transgenes and could not correlate silencing with a particular level of transgene expression. Apparently, the amount of

transgene product is not important, suggesting that the PTGS mechanism is activated in a different way. In this alternative way, the structure of the silencing T-DNA loci may play an important role. It is becoming clear that PTGS is often associated with multicopy T-DNA loci (Hobbs *et al.*, 1993; Dehio and Schell, 1994; Ingelbrecht *et al.*, 1994; Van Blokland *et al.*, 1994; English *et al.*, 1996; Stam and Kooter, unpubl. res.). It is, as yet, unknown how the organization of a transgene locus affects PTGS. There are at least two possibilities; the first is that a multicopy locus is prone to deliver the hypothetical aberrant RNA assumed to trigger the cytosolic RNA degradation machinery directly (see before). Obviously, this requires transcription of the transgenes. The aberrant nature of the RNAs might originate from read-through transcription, abnormal RNA processing, or transcription of a methylated template (Smith *et al.*, 1994; English *et al.*, 1996). When the RNA degradation machinery is activated, it eliminates all homologous RNAs. The second possibility applies only to cases where the genome contains an endogenous homologue (Fig. 1, far, right). Given that barely-transcribed *chs* sense transgenes and promoterless transgenes are able to confer strong suppression of endogenous *chs* genes (Van Blokland *et al.*, 1994; Stam and Kooter, unpubl. res.) it is unlikely, though not excluded, that a transgene-derived RNA plays a key role in this PTGS process. In line with the RdRP-cRNA model (Dougherty and Parks, 1995), we think that aberrant RNAs might be derived from the endogenous *chs* genes. But how? One possibility is that it occurs as a result of ectopic DNA pairing (Jorgensen, 1992) between the transgene locus and the endogenous gene(s). Based on the detection of elevated levels of unspliced *chs* transcripts in nuclei containing silenced *chs* genes (Van Blokland *et al.*, 1996), this pairing event may have changed features of the endogenous gene leading to an impaired processing and/or transport of the RNAs (Fig. 1). These transcripts may be intrinsically unstable and rapidly degraded, or may act as aberrant RNA causing the degradation of other homologous RNAs. Not all transgene loci may be able to pair ectopically with an endogenous gene. An essential property seems that they are repetitive. Thus far, all the T-DNA loci that we have found to induce PTGS of *chs* contain two or more T-DNAs arranged as IRs (Stam and Kooter, unpubl. res.). In the case of IR-*chs*-silencing loci, sequences near the centre of the IR are more methylated than those at the borders, and when present, the CaMV-35S promoter is barely active. This methylation pattern might be formed due to the ability of an IR to form a cruciform which initiates at the centre. A property of a methylated repeat locus is that it can induce the methylation of an ectopic transgene copy, probably by DNA-DNA pairing, and inactivate it (see before). Given this possibility, it seems reasonable to assume that a methylated IR locus is able to interact with an endogenous gene thereby modifying its epigenetic state. Thus, regarding DNA-DNA interactions, TGS and PTGS seem to have features in common. Except, that they do not lead to transcriptional silencing of the endogenous genes, probably because the homology of the transgenes is usually confined to the coding region. There are no indications that the methylation status of the endogenous genes is changed.

Observations with IRs and DRs in *Drosophila*, which lacks 5-methyl-cytidine in its DNA, indicate that repeats somehow interact with each other, leading to the formation of heterochromatin (Dorer and Henikoff, 1994). By analogy, it is conceivable that the proposed interaction between the repeat locus and the endogenous gene(s) does not lead to a change in methylation but to a change in chromatin structure. The result of this change would be the production of the hypothetical aberrant and/or unstable transcripts. By analysing petunia transformants carrying CaMV-35S promoter-driven *chs* sense or antisense transgenes, Jorgensen *et al.* (1996) showed that the pattern of *chs* silencing in flowers correlated with the repetitiveness and organization of the transgenes in these plants. The pigmentation pattern caused by single-copy transgene inserts is mostly regular (junction type) whereas that by IRs is often complex and sometimes recognizable as the 'Cossack Dancer' pattern. To explain these differences it was suggested that in silenced and unsilenced cells the transgenes are transcribed at different levels, due to the unequal distribution of transcription factors needed to express the transgenes (Jorgensen *et al.*, 1996). But, run-on assays indicate that the CaMV-35S promoter is about equally active in silenced and unsilenced cells (Van Blokland *et al.*, 1996). Thus, it seems more likely that the various silencing/pigmentation patterns are established in another fashion. Regarding the sometimes irregular silencing patterns, it is tempting to speculate that silencing is influenced by the chromatin structures of the transgenes and of the endogenous genes which may vary slightly among cells of the same tissue.

### CONCLUSIONS

The factors involved in PTGS and TGS are beginning to emerge. It seems reasonable to conclude that most cases of gene silencing involve processes acting on DNA and/or RNA in which the higher order chromatin structure of the

transgenes and endogenous genes and DNA methylation also play a role. One way of interpreting the various observations associated with gene silencing is that there are several independent pathways leading to gene silencing. Alternatively, one can look at the different observations as representing different steps of the same pathway, which contains side branches and entry points as shown in Fig. 3. In this working model, the most up-stream step is the presence of a multi-copy transgene locus and the most down-stream one is the hypothetical cRNA-mediated RNA degradation process. Methylation and/or heterochromatinization of transgenes may result in transcriptional inactivation. If methylation does not lead to transcriptional silencing, transcription may give rise to aberrant RNAs, thereby activating the RdRP-cRNA mechanism. If RdRP is involved, it is important to find out what RNA templates are used. Is it indeed the hypothetical aberrant RNA that results from RNA over-abundance, from RNA-modification, from a particular transgene configuration, or from an endogenous gene as a result of *trans*-interactions with a multicopy transgene locus?

The RdRP-cRNA model makes several testable predictions, including the production of cRNAs. However, a crucial test is to generate mutants in which the RdRP gene(s) are knocked-out and to see whether these plants, if viable, are able to induce the PTGS mechanism. This awaits cloning of the RdRP genes. Another genetic approach is to identify mutants in which silencing is changed by mutations in host factor genes. Two such modifier loci have been genetically identified in *Arabidopsis* which affect PTGS of *rolB* transgenes (Dehio and Schell, 1994). Once we have answered some of the questions regarding the mechanisms of silencing we may be able to design transgene constructs that efficiently silence endogenous genes. It should also be possible to prevent silencing of transgenes, if stable expression is required. In this regard, considerable progress has already been made by flanking transgenes with matrix associated regions (MARs; Mlynarova *et al.*, 1994, 1995; Spiker and Thompson, 1996). Besides these practical considerations, the exciting part of the silencing research is the disclosure of some intriguing cellular processes in plants. It will be a challenge to learn if they play a regular role in controlling gene expression or if they act as a defence response to the effects of invading DNA, virus infections, transposon activity or DNA rearrangements.

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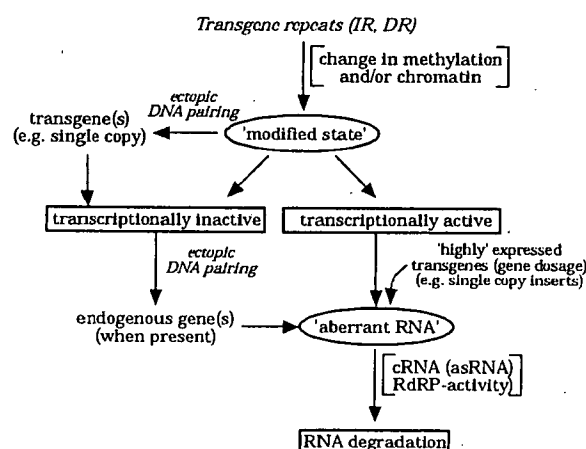


FIG. 3. Gene silencing associated phenomena viewed as different features of one and the same pathway. The most upstream feature is the presence of a multicopy transgene locus, the most downstream, the activation of a sequence-specific RNA degradation process (see text for further details).



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**United States Patent** [19]  
**Brown et al.****[11] Patent Number: 5,859,347**  
**[45] Date of Patent: \*Jan. 12, 1999****[54] ENHANCED EXPRESSION IN PLANTS****[75] Inventors:** Sherri Marie Brown, Chesterfield;  
Colleen Gail Santino, St. Louis, both  
of Mo.**[73] Assignee:** Monsanto Company, St. Louis, Mo.**[\*] Notice:** The term of this patent shall not extend  
beyond the expiration date of Pat. Nos.  
5,593,874 and 5,424,412.**[21] Appl. No.: 758,149****[22] Filed: Nov. 25, 1996****Related U.S. Application Data****[63]** Continuation of Ser. No. 333,665, Nov. 3, 1994, Pat. No.  
5,593,874, which is a continuation of Ser. No. 181,364, Jan.  
13, 1994, Pat. No. 5,424,412, which is a continuation of Ser.  
No. 855,857, Mar. 19, 1992, abandoned.**[51] Int. Cl.<sup>6</sup> ..... A01H 5/00; C12N 15/11****[52] U.S. Cl. .... 800/205; 536/23.1; 536/24.1;**  
435/172.3; 435/69.1; 435/70.1; 435/320.1**[58] Field of Search .... 800/205, DIG. 55,**  
800/DIG. 56, DIG. 57, DIG. 58; 536/23.1,  
24.1; 435/172.3, 69.1, 70.1, 320.1**[56] References Cited****U.S. PATENT DOCUMENTS**5,424,412 6/1995 Brown et al. .... 800/205  
5,593,874 1/1997 Brown et al. .... 800/205*Primary Examiner*—Elizabeth C. Kemmerer  
*Attorney, Agent, or Firm*—Arnold, White & Durkee**[57] ABSTRACT**This invention provides HSP70 introns that when present in  
a non-translated leader of a chimeric gene enhance expres-  
sion in plants.**35 Claims, 28 Drawing Sheets**

1 AGATCTACCG TCTTCGGTAC GCGCTCACTC CGCCCTCTGC CTTTGTTACT  
51 GCCACGTTTC TCTGAATGCT CTCTTGTTGTG GTGATTGCTG AGAGTGGTTT  
101 AGCTGGATCT AGAATTACAC TCTGAAATCG TGTTCTGCCT GTGCTGATTA  
151 CTTGCCGTCC TTTGTAGCAG CAAAATATAG GGACATGGTA GTACGAAACG  
201 AAGATAGAAC CTACACAGCA ATACGAGAAA TGTGTAATTT GGTGCTTAGC  
251 GGTATTTATT TAAGCACATG TTGGTGTTAT AGGGCACTTG GATTCAGAAG  
301 TTTGCTGTTA ATTTAGGCAC AGGCTTCATA CTACATGGGT CAATAGTATA  
351 GGGATTCATA TTATAGGCGA TACTATAATA ATTTGTTTCGT CTGCAGAGCT  
401 TATTATTTGC CAAAATTAGA TATTCCTATT CTGTTTTTGT TTGTGTGCTG  
451 TTAAATTGTT AACGCCTGAA GGAATAAATA TAAATGACGA AATTTTGATG  
501 TTTATCTCTG CTCCTTTATT GTGACCATAA GTCAAGATCA GATGCACTTG  
551 TTTTAAATAT TGTTGTCTGA AGAAATAAGT ACTGACAGTA TTTTGATGCA  
601 TTGATCTGCT TGTTTGTTGT AACAAAATTT AAAAATAAAG AGTTTCCTTT  
651 TTGTTGCTCT CCTTACCTCC TGATGGTATC TAGTATCTAC CAACTGACAC  
701 TATATTGCTT CTCTTTACAT ACGTATCTTG CTCGATGCCT TCTCCCTAGT  
751 GTTGACCAGT GTTACTCACA TAGTCTTTGC TCATTTTCATT GTAATGCAGA  
801 TACCAAGCGG CCATGG

FIGURE 1

1	AGATCTACCG	TCTTCGGTAC	GGGCTCACTC	CGCCCTCTGC	CTTTGTTACT
51	GCCACGTTTC	TCTGAATGTG	ATCTGCTTGT	TTGTTGTAAC	AAAATTTAAA
101	AATAAAGAGT	TTCCTTTTTG	TTGCTCTCCT	TACCTCCTGA	TGGTATCTAG
151	TATCTACCAA	CTGACACTAT	ATTGCTTCTC	TTTACATACG	TATCTTGCTC
201	GATGCCTTCT	CCCTAGTGTT	GACCAGTGTT	ACTCACATAG	TCTTTGCTCA
251	TTTCATTGTA	ATGCAGATAC	CAAGCGGCCA	TGG	

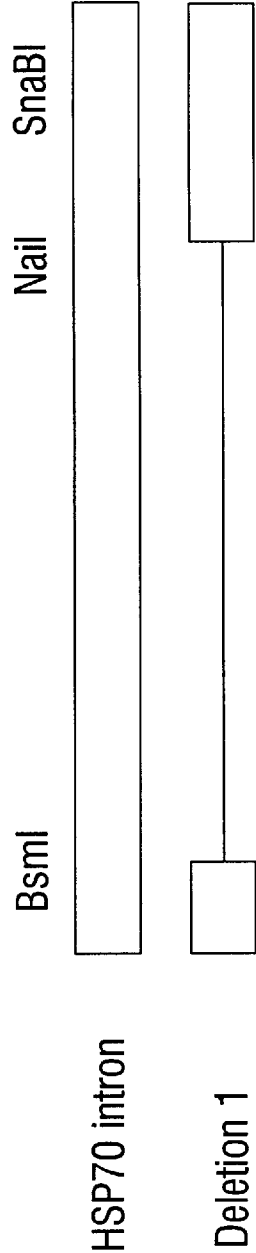


FIG. 2

1	AGATCTACCG	TCTTCGGTAC	GGCTCACTC	CGCCCTCTGC	CTTTGTTACT
51	GCCACGTTTC	TCTGAATGGT	ATCTTGCTCG	ATGCCTTCTC	CCTAGTGTTC
101	ACCAGTGTTA	CTCACATAGT	CTTTGCTCAT	TTCATTGTAA	TGCAGATACC
151	AAGCGGCCAT	GG			

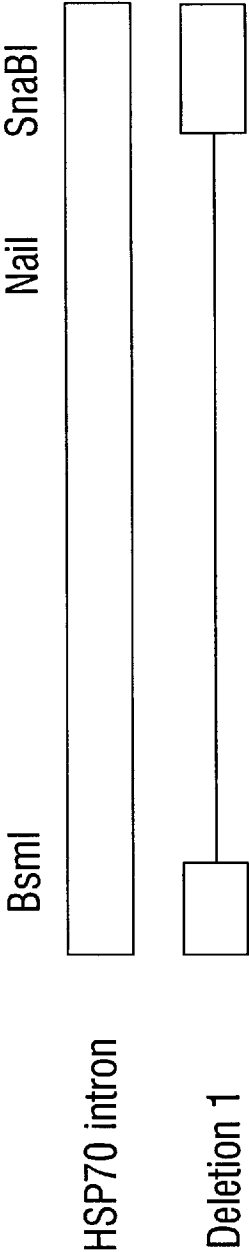
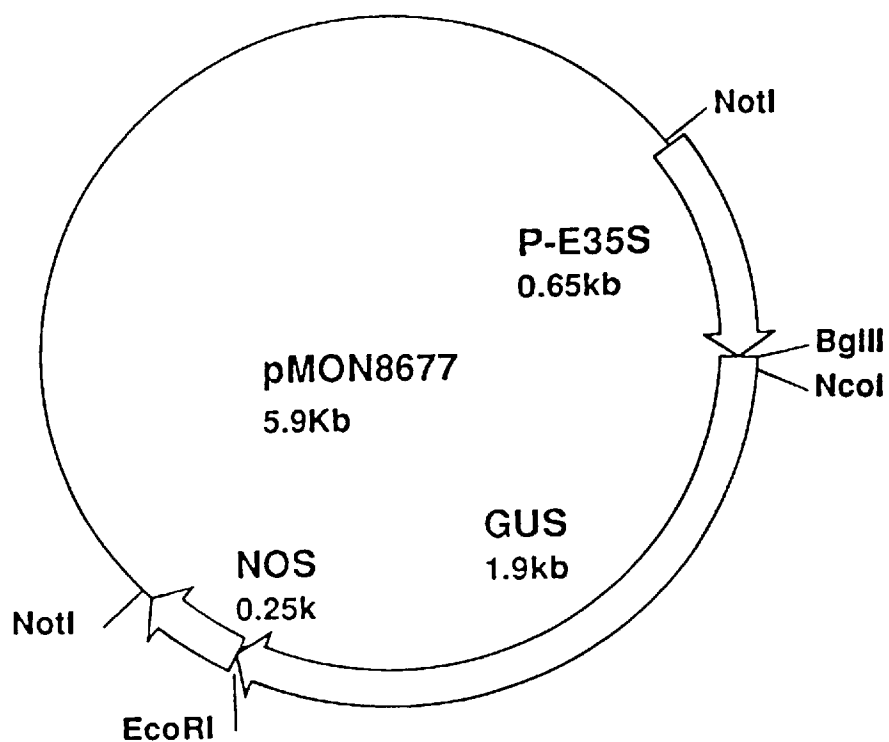
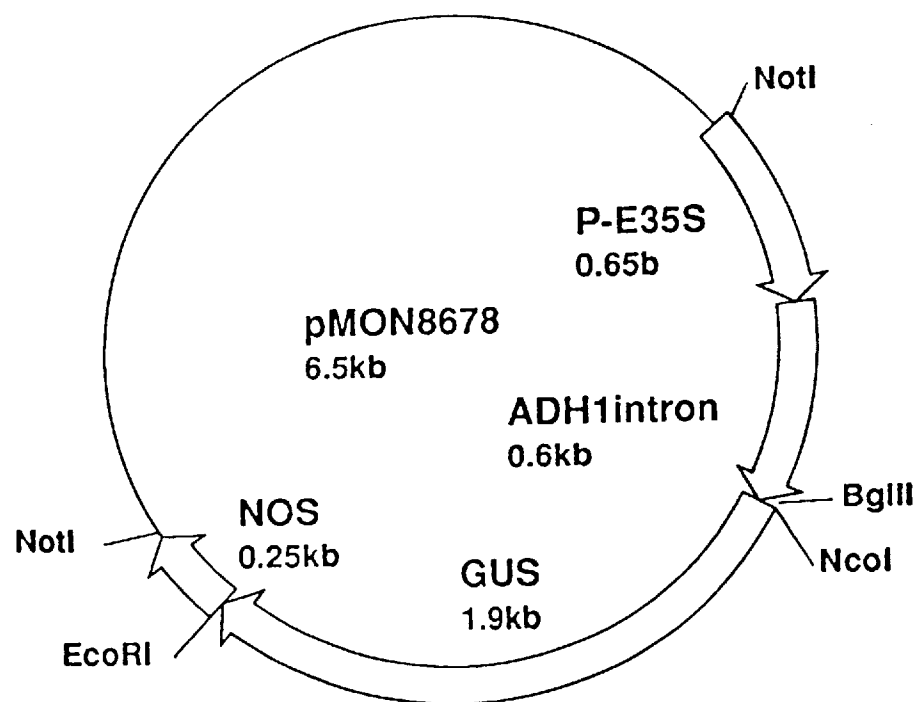


FIG. 3

**Figure 4**

**Figure 5**



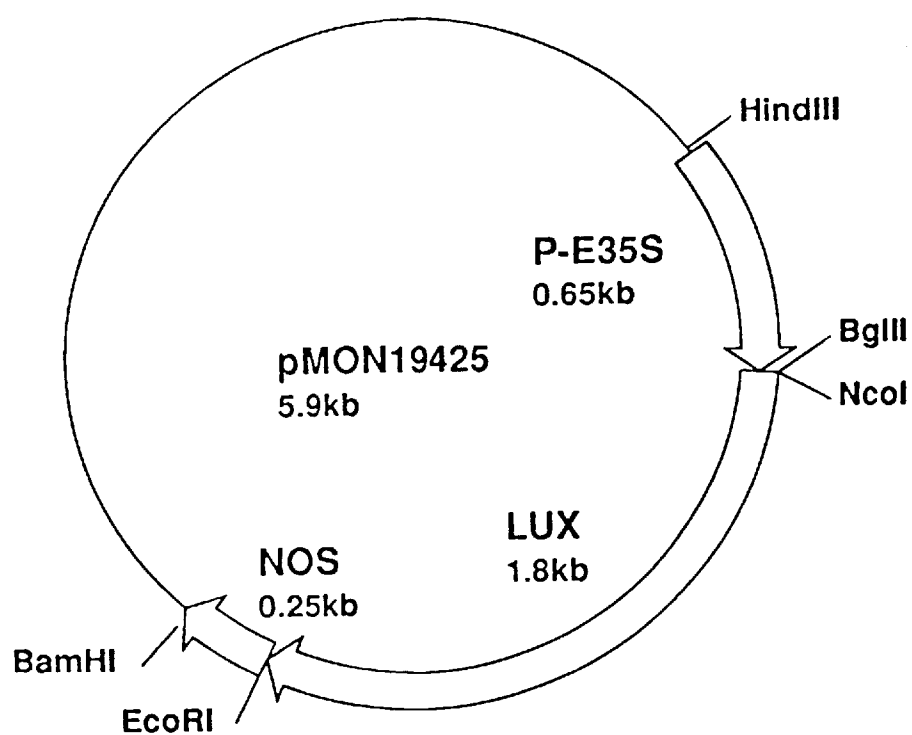


Figure 6

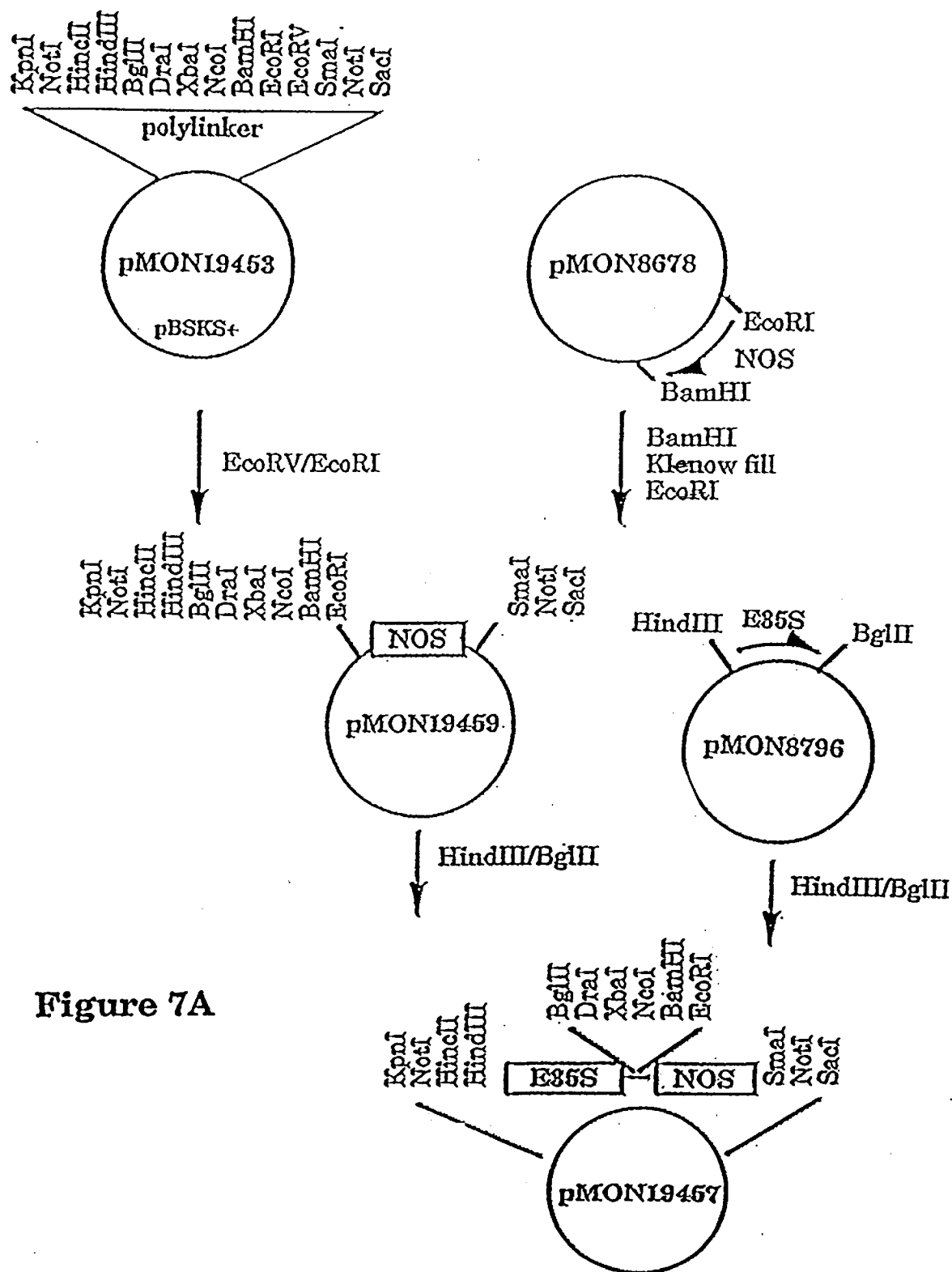
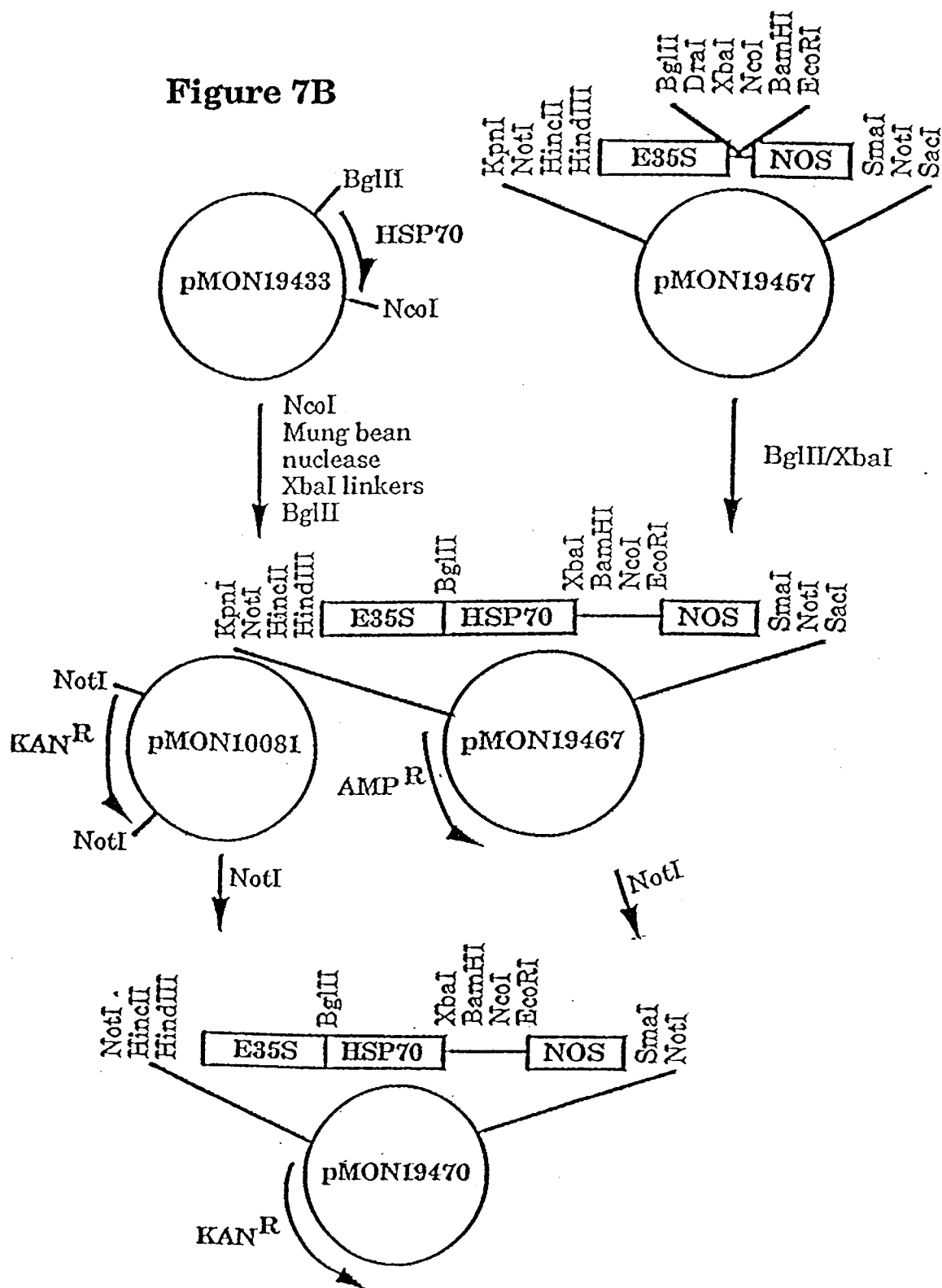


Figure 7A

Figure 7B



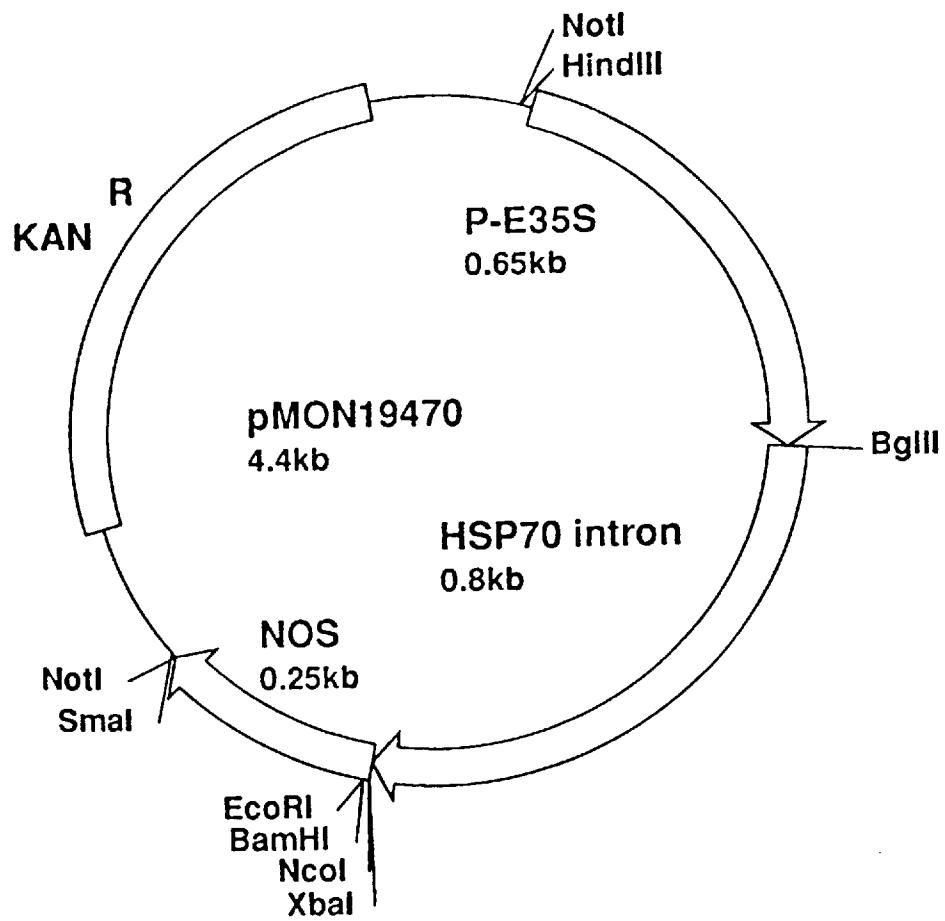


Figure 8

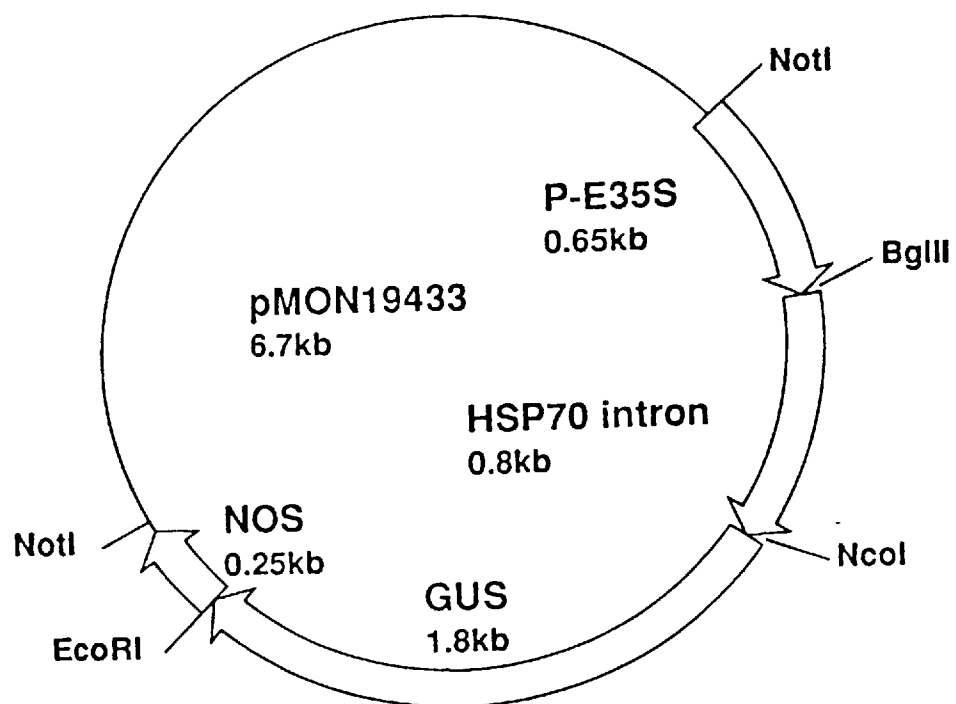


Figure 9

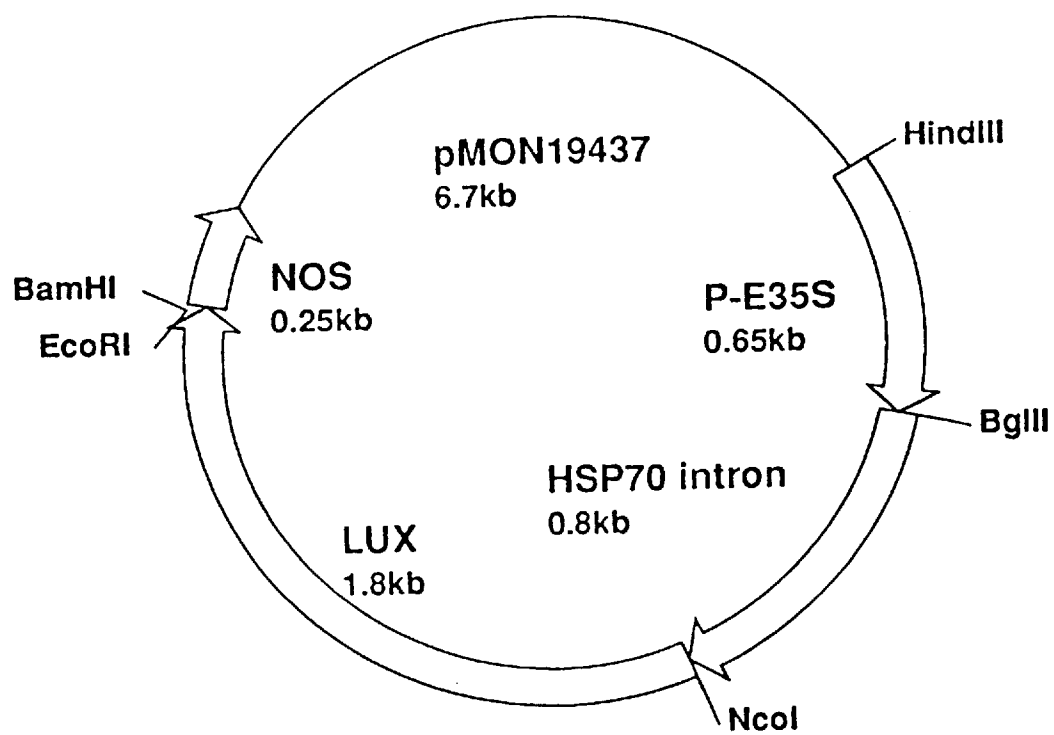


Figure 10

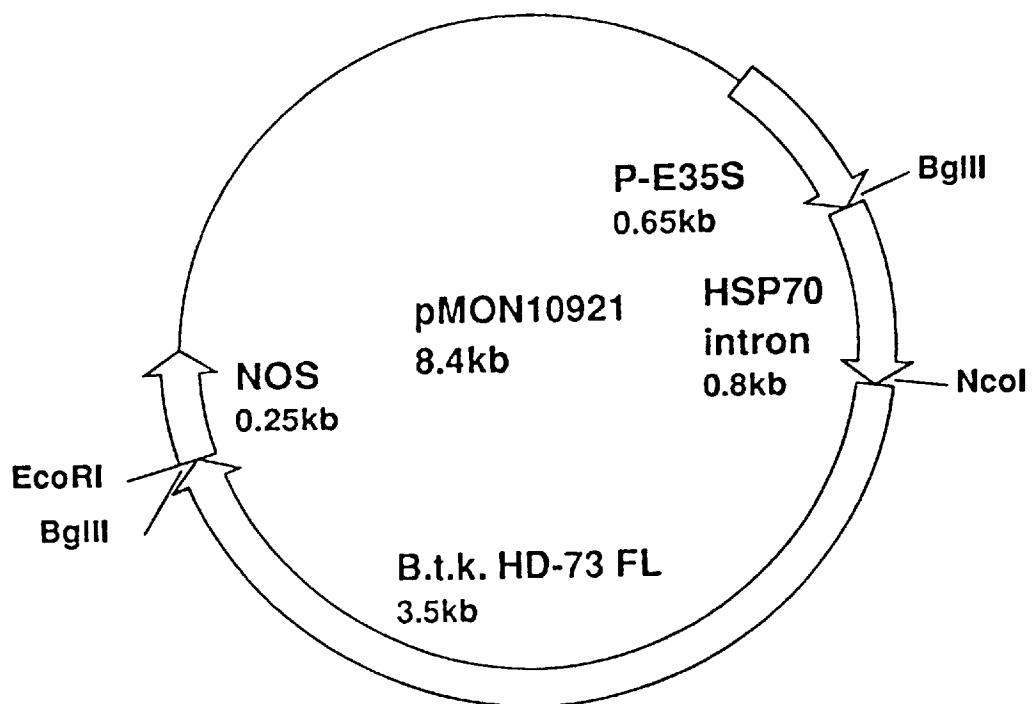
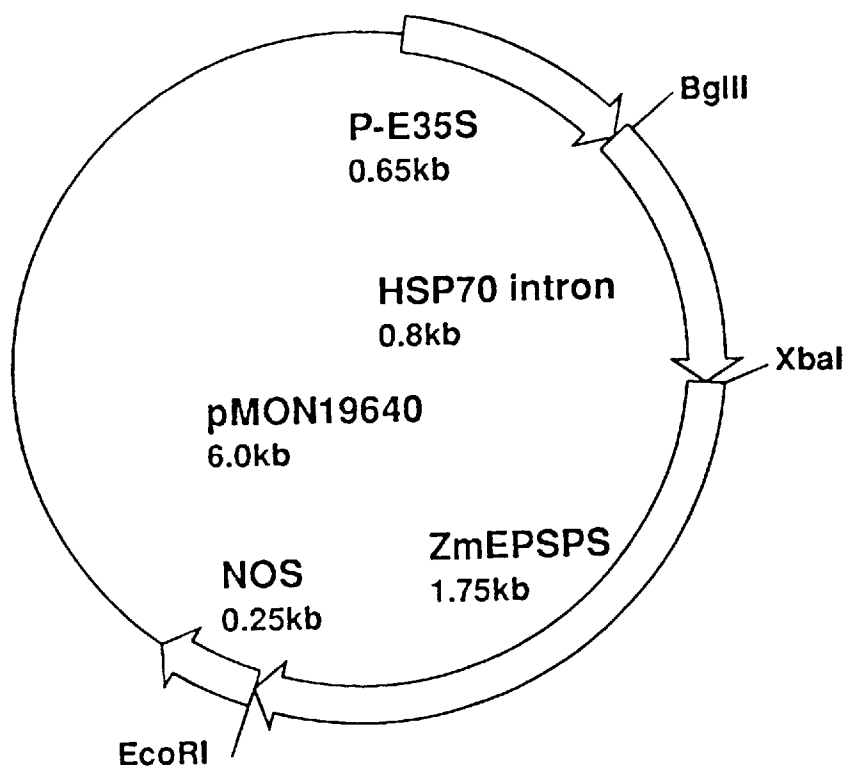


Figure 11

**Figure 12**



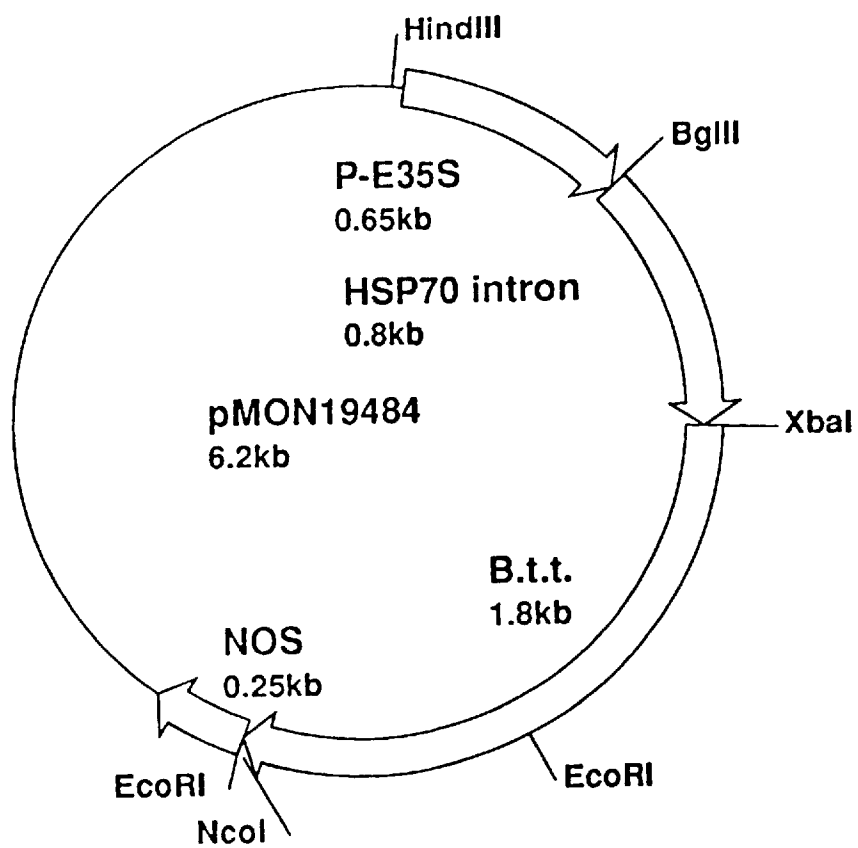
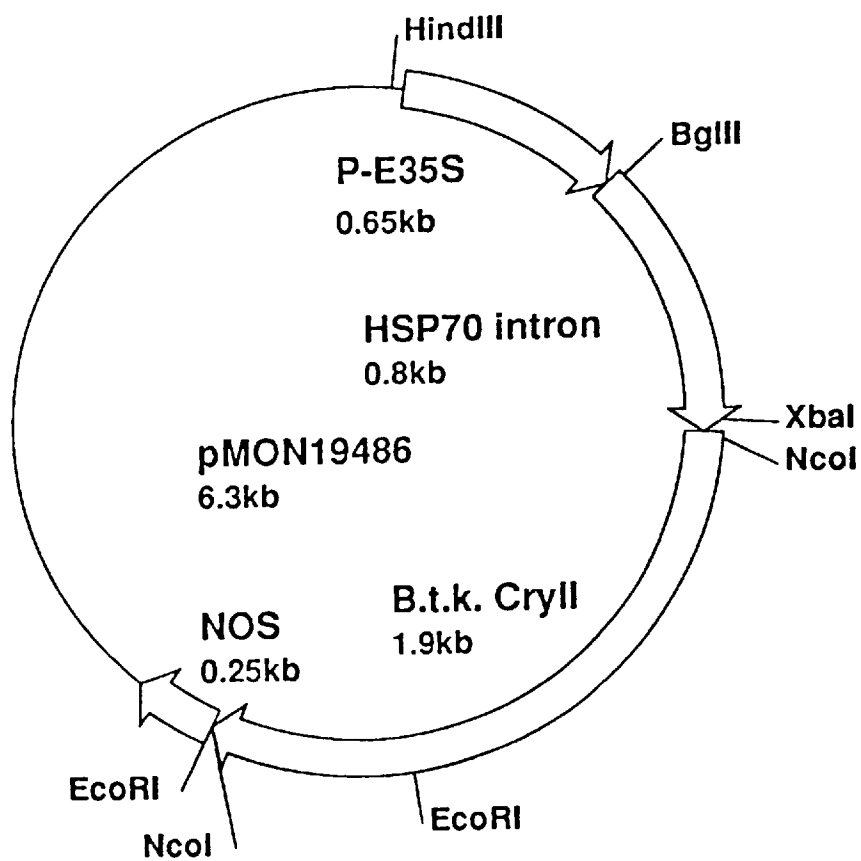
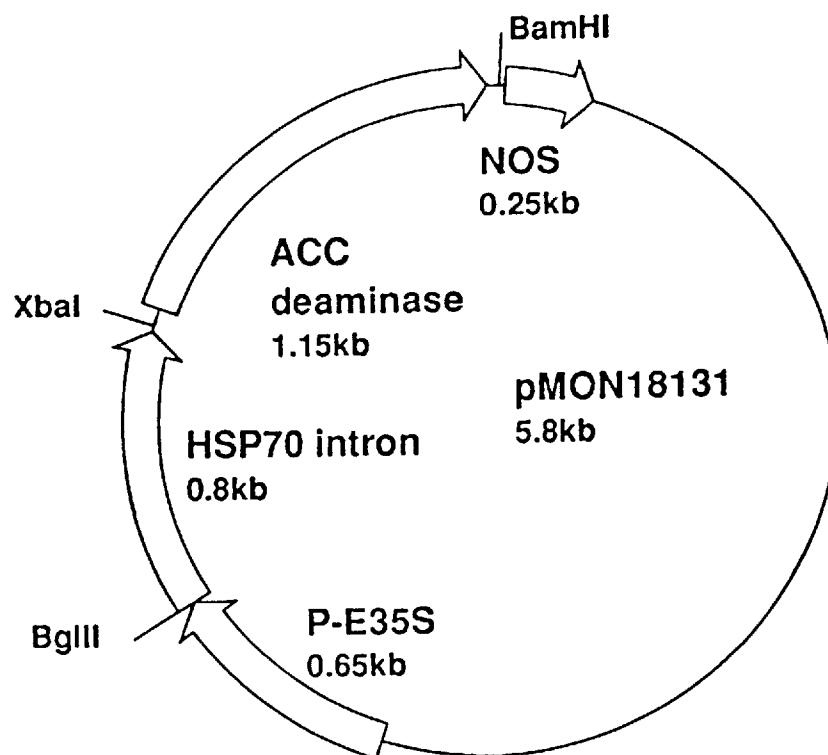


Figure 13

**Figure 14**

**Figure 15**

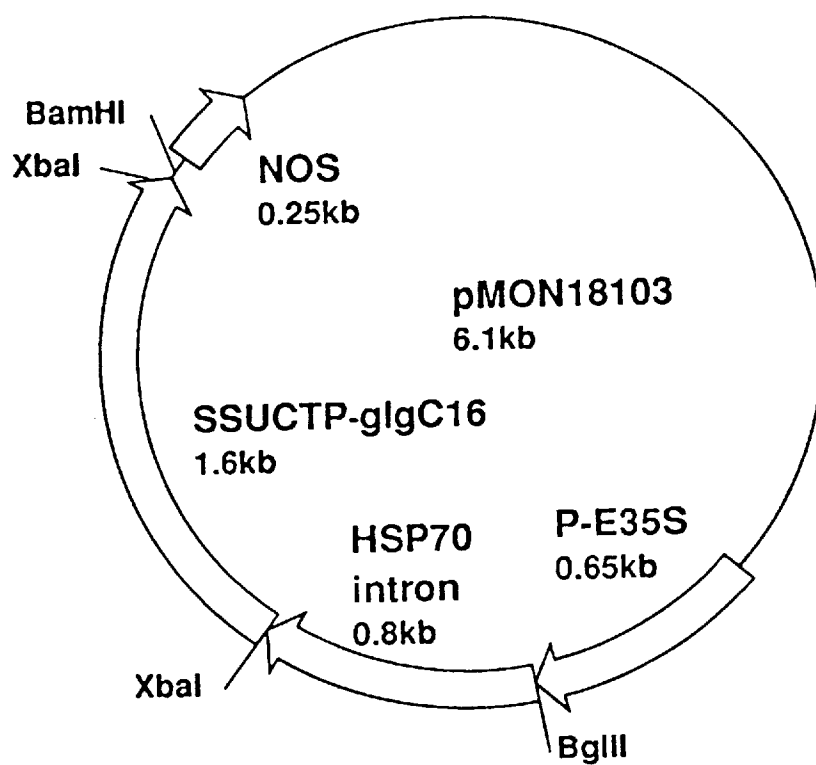


Figure 16

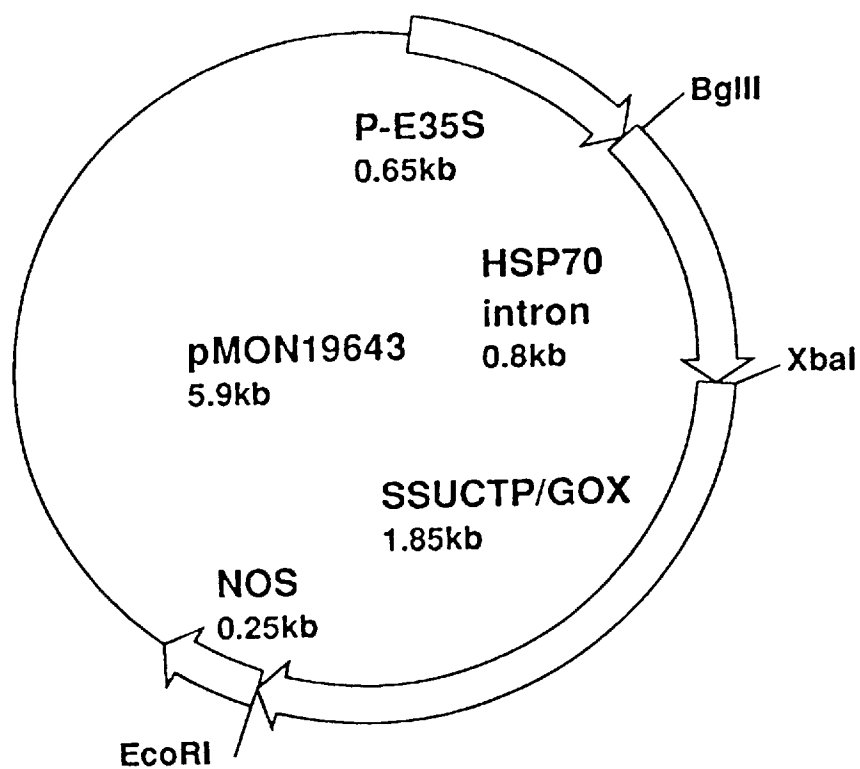


Figure 17

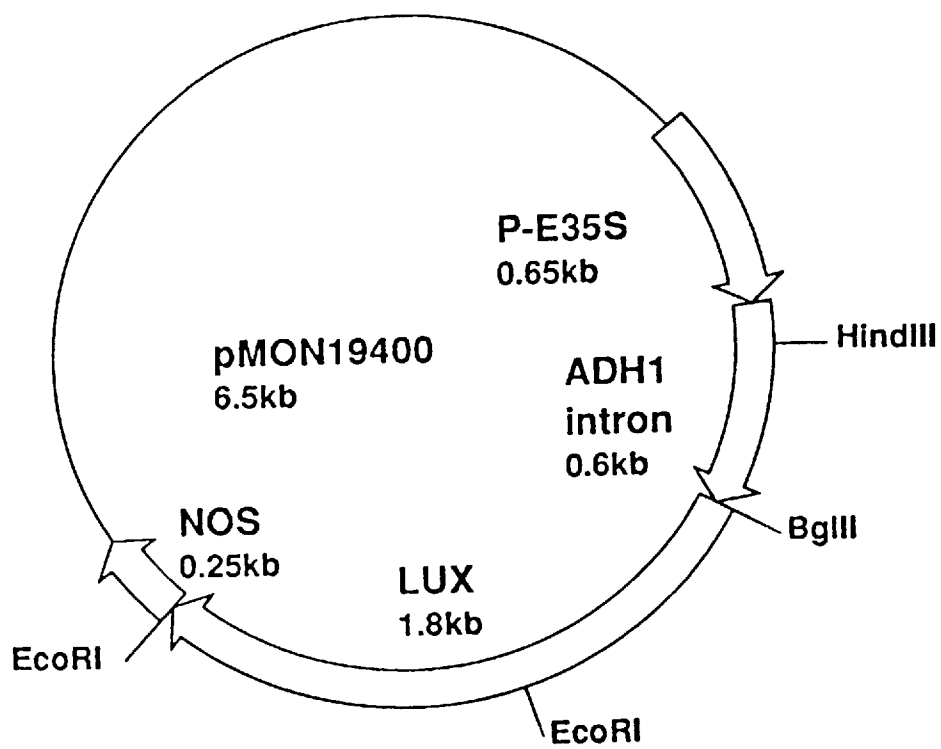


Figure 18

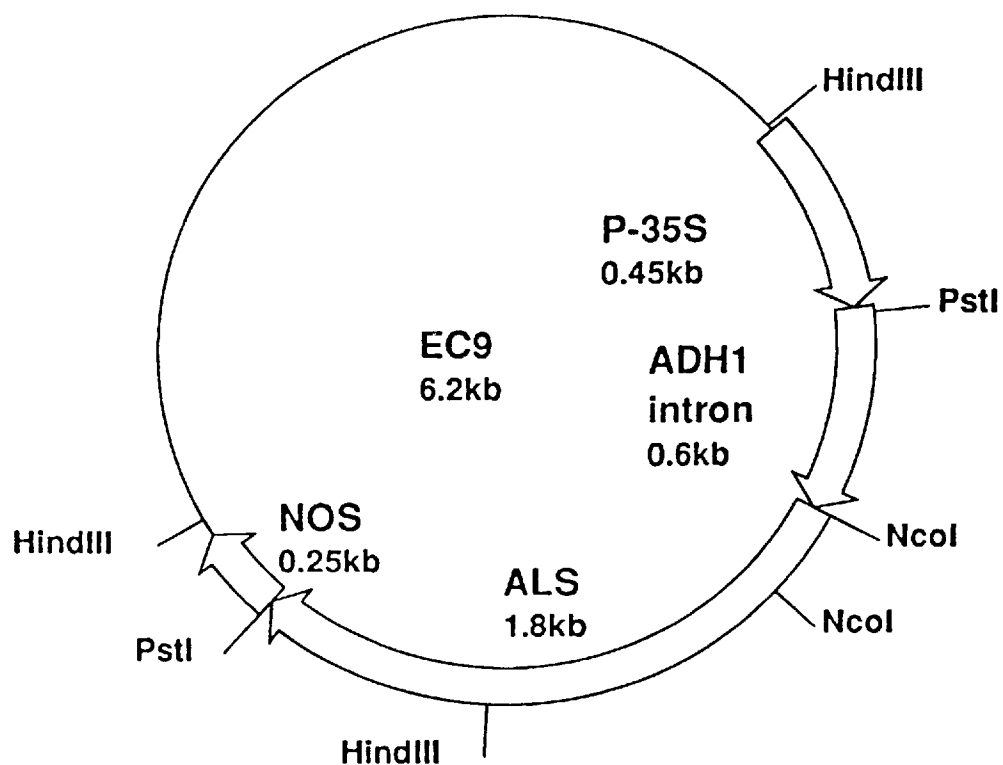
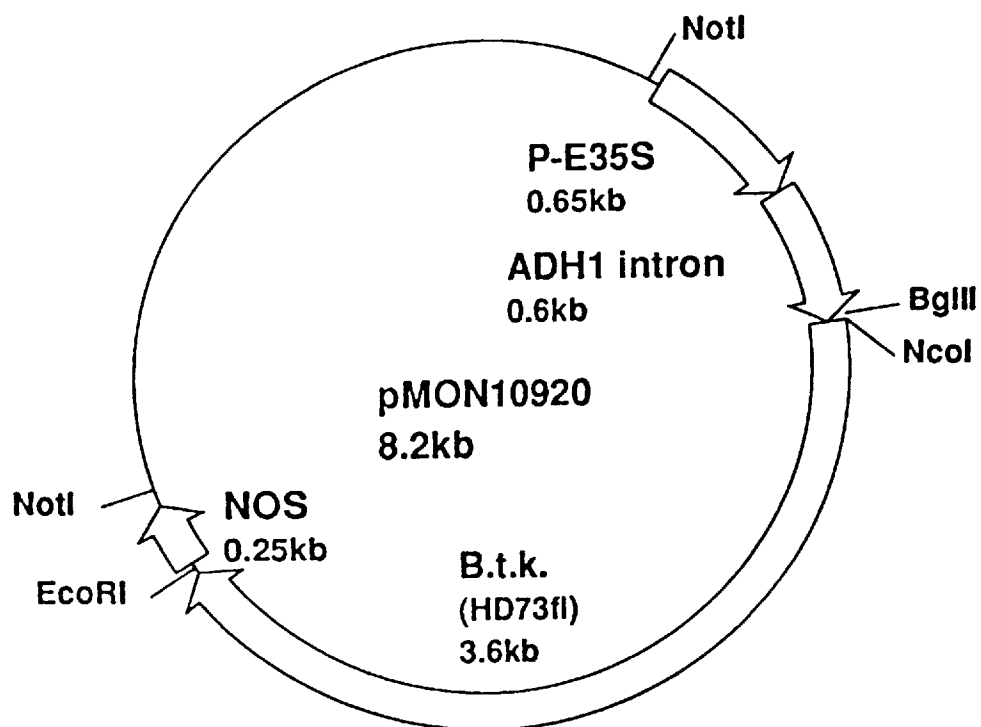


Figure 19

**Figure 20**



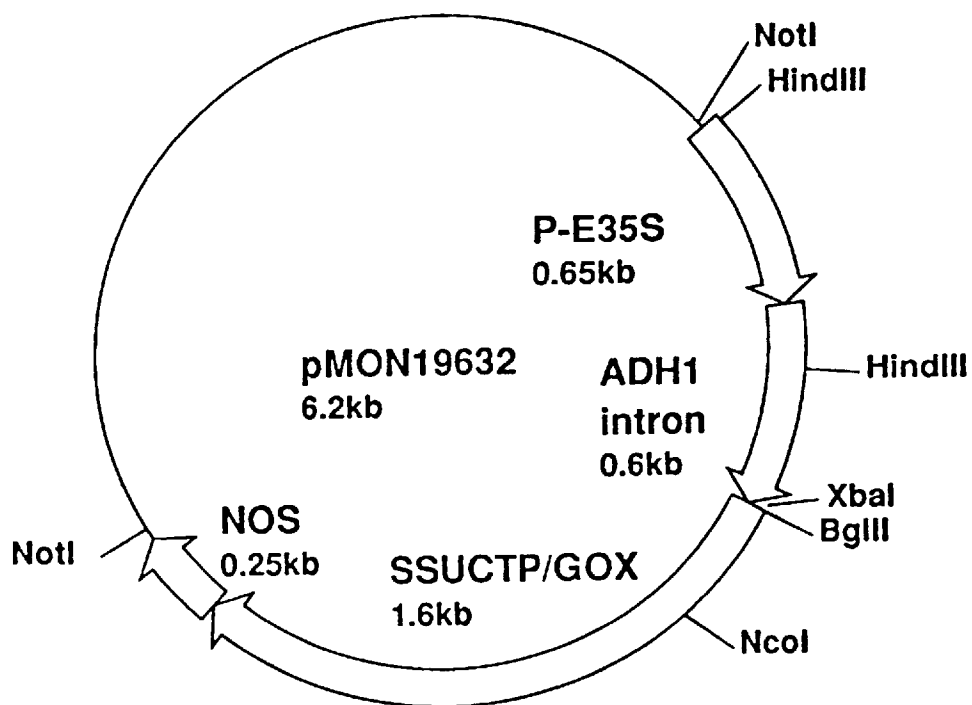
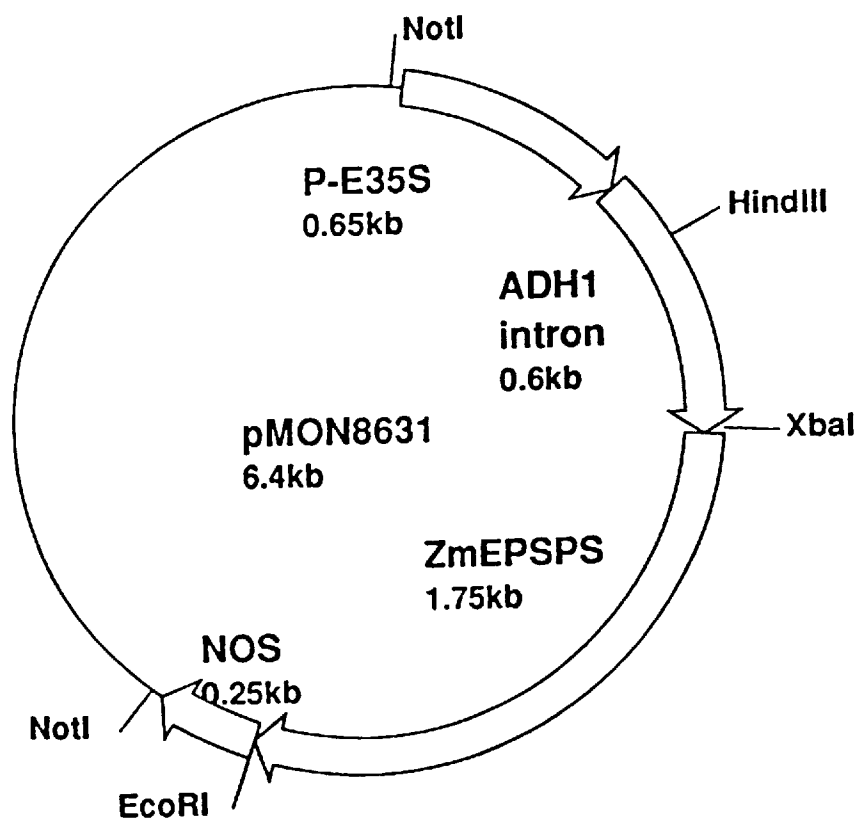
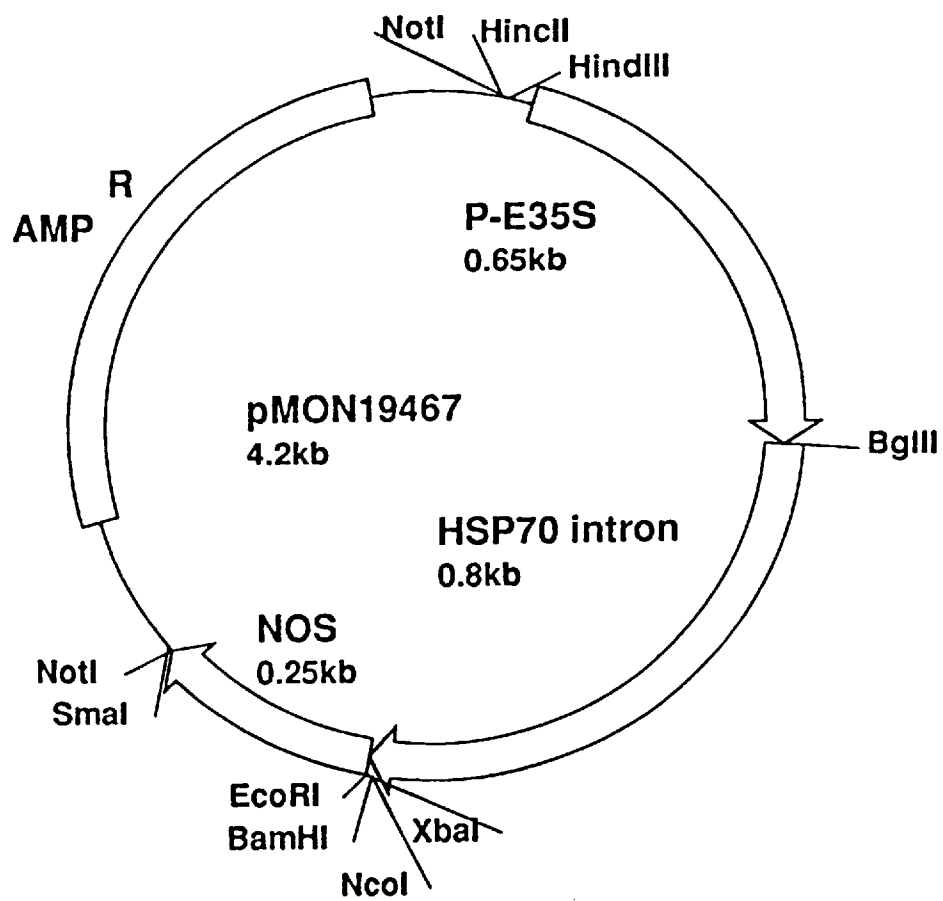
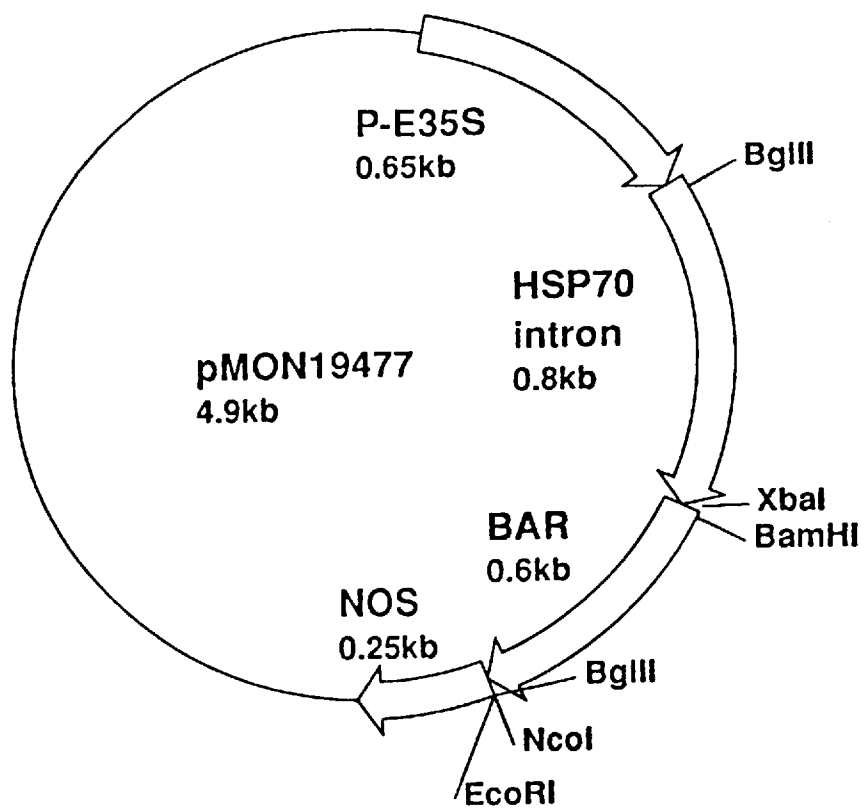
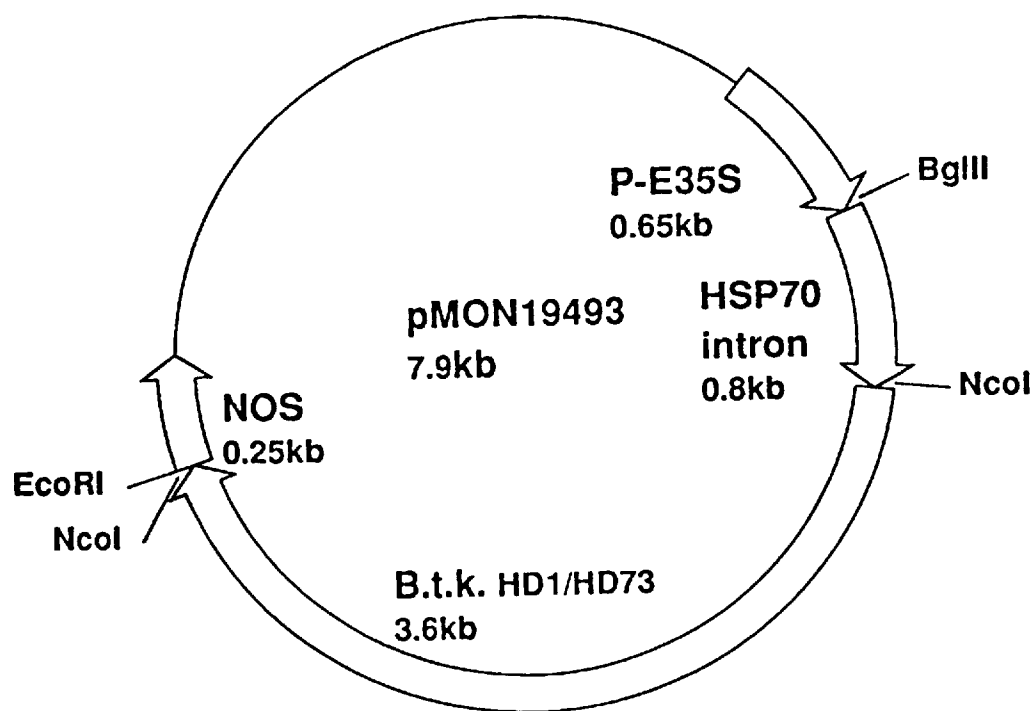


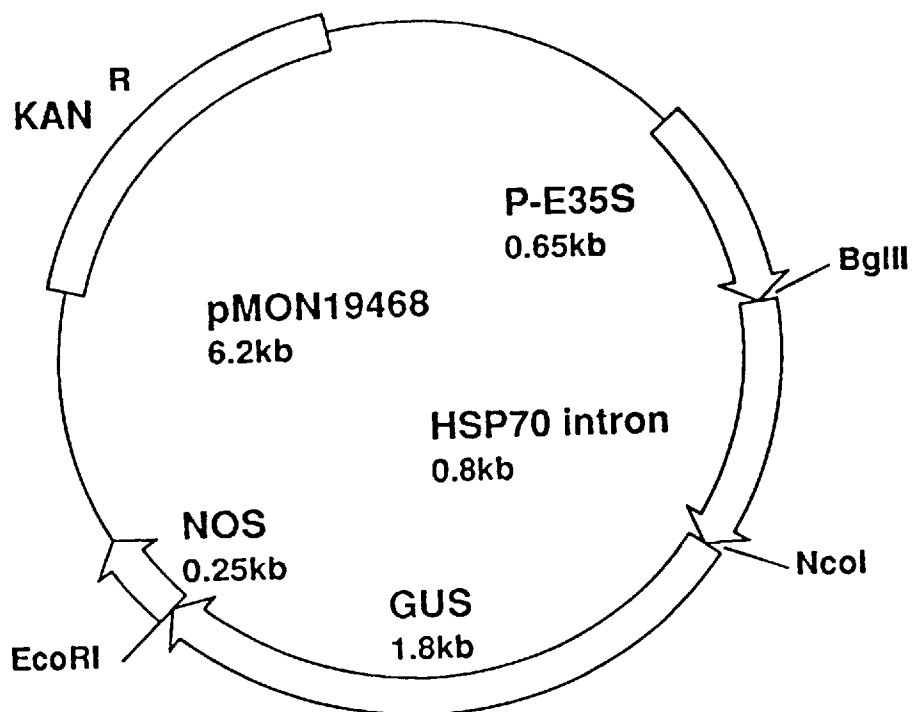
Figure 21

**Figure 22**

**Figure 23**

**Figure 24**

**Figure 25**

**Figure 26**

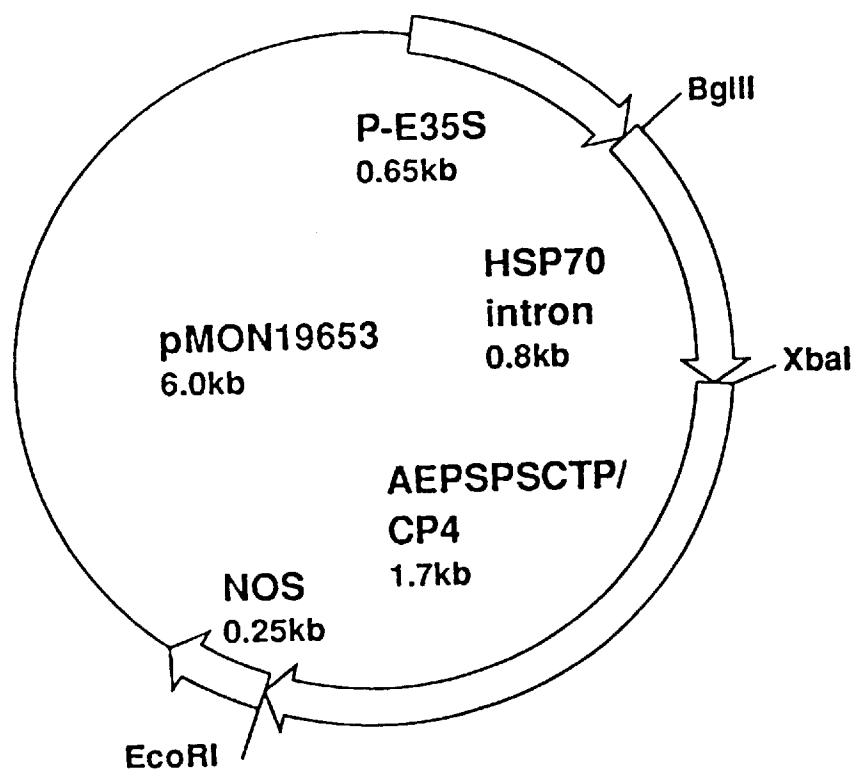


Figure 27

## ENHANCED EXPRESSION IN PLANTS

This is a continuation of application Ser. No. 08/333,665 filed Nov. 3, 1994 which issued as U.S. Pat. No. 5,593,874 on Jan. 14, 1987; which is a continuation of application Ser. No. 08/181,364 filed Jan. 13, 1994 which issued as U.S. Pat. No. 5,424,412 on Jun. 13, 1995; which is a continuation of application Ser. No. 07/855,857 filed on Mar. 19, 1992, now abandoned.

This invention relates to recombinant expression systems, particularly to plant expression systems for expressing greater quantities of proteins in plants.

### BACKGROUND OF THE INVENTION

Recombinant genes for producing proteins in plants comprise in sequence a promoter which functions in plants, a structural gene encoding the target protein, and a non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the RNA sequence. Much scientific effort has been directed to improve these recombinant plant genes to express larger amounts of the target protein.

One advantage of higher levels of expression is that fewer numbers of transgenic plants need to be produced and screened to recover plants producing sufficient quantities of the target protein to be agronomically significant. High level expression leads to plants exhibiting commercially important phenotypical properties.

Improved recombinant plant genes have been found by use of more potent promoters, such as promoters from plant viruses. Further improvement in expression has been obtained in gene constructs by placing enhancer sequences 5' the promoter. Still further improvement has been achieved, especially in monocot plants by gene constructs having introns in the non-translated leader positioned between the promoter and the structural gene coding sequence. For example, Callis et al. (1987) *Genes and Development*, Vol. 1, pp. 1183-1200, reported that the presence of alcohol dehydrogenase-1 (Adh-1) introns or Bronze-1 introns resulted in higher levels of expression. Dietrich et al. (1987) *J. Cell Biol.*, 105, p. 67, reported that the 5' untranslated leader length was important for gene expression in protoplast. Mascarenhas et al. (1990) *Plant Mol. Biol.*, Vol. 15, pp. 913-920, reported a 12-fold and 20-fold enhancement of CAT expression by use of the Adh-1 intron. Vasil et al. (1989) *Plant Physiol.*, 91, pp. 1575-1579, reported that the Shrunken-1 (Sh-1) intron gave about 10 times higher expression than constructs containing the Adh-1 intron. Silva et al. (1987) *J. Cell Biol.*, 105, p. 245, reported a study of the effect of the untranslated region of the 18 Kd heat shock protein (HSP18) gene on expression of CAT. Semrau et al. (1989) *J. Cell Biol.*, 109, p. 39A, and Mettler et al., N.A.T.O. Advanced Studies Institute on Molecular Biology, Elmer, Bavaria (May 1990) reported that the 140 bp intron of the 82 Kd heat shock protein (HSP82) enhanced expression in maize protoplasts.

The search for even more improved recombinant plant genes continues for the reasons discussed above.

### SUMMARY OF THE INVENTION

This invention is for an improved method for the expression of a chimeric plant gene in plants, particularly to achieve higher expression in monocot plants. The improvement of the invention comprises expressing a chimeric plant gene with an intron derived from the 70 Kd maize heat shock protein (HSP70) selected from the group consisting essen-

tially of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 in the non-translated leader positioned 3' from the gene promoter and 5' from the structural DNA sequencing encoding a protein.

One embodiment of the invention is a recombinant, double stranded DNA molecule comprising in sequence:

- (a) a promoter that functions in plant cells to cause the production of an RNA sequence;
- (b) a non-translated leader DNA sequence comprising an intron selected from the group consisting essentially of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3;
- (c) a structural DNA sequence that causes the production of an RNA sequence that encodes a protein; and
- (d) a 3' non-translated sequence that functions in plant cells to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence, the intron being heterologous with respect to the promoter.

Another embodiment of the invention is an isolated DNA sequence consisting essentially of the nucleotides shown in SEQ ID NO:1.

Another embodiment of the invention is a synthetic DNA sequence selected from the group consisting essentially of the nucleotides shown in SEQ ID NO:2 and nucleotides shown in SEQ ID NO:3.

Another embodiment of the invention is transgenic plants, particularly monocot plants, comprising the chimeric plant genes described above. The resultant transgenic plants are capable of expressing a foreign gene which has been inserted into the chromosome of the plant cell.

The invention provides chimeric plant genes that, when expressed in a transgenic plant, provide greater quantities of the desired protein encoded by the structural coding sequence in the chimeric gene of the invention. The high protein levels impart important agronomic properties to the plant depending on which protein is present. For example, expression of a *Bacillus thuringiensis* crystal toxin protein protects the transgenic plant from insect attack. Expression of a plant virus coat protein protects the transgenic plant from plant viral infections. Expression of a glyphosate tolerant gene protects the transgenic plant from the herbicidal action of glyphosate herbicide.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the DNA sequence of the intron from the 70 Kd maize heat shock protein, SEQ ID NO:1.

FIG. 2 illustrates a truncated DNA sequence with internal deletions of the intron from the 70 Kd maize heat shock protein, SEQ ID NO:2.

FIG. 3 illustrates another truncated DNA sequence with internal deletions of the intron from the 70 Kd maize heat shock protein, SEQ ID NO:3.

FIG. 4 illustrates a physical map of the plasmid pMON8677.

FIG. 5 illustrates a physical map of the plasmid pMON8678.

FIG. 6 illustrates a physical map of the plasmid pMON19425.

FIG. 7A shows the steps employed to prepare pMON19457. FIG. 7B shows the steps employed to prepare pMON19470.

FIG. 8 illustrates a physical map of the plasmid pMON19470 comprising the HSP70 intron and a number of restriction sites for insertion of a structural gene encoding a protein to be expressed in plants.

FIG. 9 illustrates a physical map of the plasmid pMON19433 comprising an HSP70 intron and a GUS coding sequence.



FIG. 10 illustrates a physical map of the plasmid pMON19437 comprising an HSP70 intron and a LUX coding sequence.

FIG. 11 illustrates a physical map of the plasmid pMON10921 comprising an HSP70 intron and a B.t.k.-HD73 coding sequence.

FIG. 12 illustrates a physical map of the plasmid pMON19640 comprising an HSP70 intron and an EPSPS:215 coding sequence.

FIG. 13 illustrates a physical map of the plasmid pMON19484 comprising an HSP70 intron and a B.t.t. coding sequence.

FIG. 14 illustrates a physical map of the plasmid pMON19486 comprising an HSP70 intron and a B.t.k.-P2 CryII coding sequence.

FIG. 15 illustrates a physical map of the plasmid pMON18131 comprising an HSP70 intron and an ACC-deaminase coding sequence.

FIG. 16 illustrates a physical map of the plasmid pMON18103 comprising a truncated HSP70 intron and a glgC16 coding sequence.

FIG. 17 illustrates a physical map of the plasmid pMON18104 comprising an HSP70 intron and a GOX coding sequence.

FIG. 18 illustrates a physical map of the plasmid pMON19643 comprising the HSP70 intron and the LUX coding sequence.

FIG. 19 illustrates a physical map of the plasmid EC9 comprising the ADH1 intron.

FIG. 20 illustrates a physical map of the plasmid pMON10920 comprising a B.t.k. coding sequence—HD73 full length.

FIG. 21 illustrates a physical map of the plasmid pMON19632 comprising an ADH1 intron and a GOX coding sequence.

FIG. 22 illustrates a physical map of the plasmid pMON8631 comprising a maize EPSPS coding sequence.

FIG. 23 illustrates a physical map of the cassette plasmid pMON19467 comprising an HSP70 intron.

FIG. 24 illustrates a physical map of the plasmid pMON19477 comprising a BAR coding sequence.

FIG. 25 illustrates a physical map of the plasmid pMON19493 comprising a B.t.k. coding sequence—HD1/HD73 hybrid.

FIG. 26 illustrates a physical map of the plasmid pMON19648 comprising a GUS coding sequence.

FIG. 27 illustrates a physical map of the plasmid pMON19653 comprising a CP4 coding sequence.

#### DETAILED DESCRIPTION OF THE INVENTION

The intron of the chimeric gene of this invention was derived using the polymerase chain reaction (PCR) from the 70 Kd maize heat shock protein (HSP70) in pMON9502 described by Rochester et al. (1986) *Embo. J.*, 5:451–458. The intron sequence disclosed herein (SEQ ID NO:1) contains 773 base pair HSP70 intron with 10 base pairs of flanking 5' exon sequence and 11 base pairs of flanking 3' exon sequence. The primers used to isolate the intron are designed such that the PCR product contains a 6 base pair BglII site at the 5' end and a 6 base pair NcoI site at the 3' end.

Chimeric genes are constructed by inserting the intron into BglII and NcoI sites in the 5' non-translated leader of an

expression vector comprising a plant promoter, a scorable marker coding sequence, and a polyadenylated coding sequence. The expression vectors are constructed with the appropriate restriction sites which permit the insertion of a structural DNA sequence encoding the desired protein. Conventional cloning and screening procedures are used throughout unless otherwise noted.

A gene of this invention containing the HSP70 intron can be inserted into a suitable plant transformation vector for transformation into the desired plant species. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*. A plant transformation vector preferably includes all of the necessary elements needed for transformation of plants or plant cells. Typical plant transformation vectors comprise selectable marker genes, one or both of the T-DNA borders, cloning sites, appropriate bacterial genes to facilitate identification of transconjugates, broad host-range replication and mobilization functions and other elements as desired.

Transformation of plant cells may be effected by delivery of a transformation vector or of free DNA by use of a particle gun which comprises directing high velocity micro-projectiles coated with the vector or DNA into plant tissue. Selection of transformed plant cells and regeneration into whole plants may be carried out using conventional procedures. Other transformation techniques capable of inserting DNA into plant cells may be used, such as electroporation or chemicals that increase free DNA uptake.

The HSP70 intron cDNA sequence is inserted into a plant transformation vector as a gene capable of being expressed in a plant. For the purposes of this invention, a “gene” is defined as an element or combination of elements that are capable of being expressed in a plant, either alone or in combination with other elements. Such a gene generally comprises, in the following order, a promoter that functions in plant cells, a 5' non-translated leader sequence, a DNA sequence coding for the desired protein, and a 3' non-translated region that functions in plants to cause the addition of polyadenylated ribonucleotides to the 3' end of the mRNA transcript. In this definition, each above described element is operationally coupled to the adjacent element. A plant gene comprising the above elements can be inserted by known, standard recombinant DNA methods into a plant transformation vector and other elements added to the vector when necessary. A plant transformation vector can be prepared that has all of the necessary elements for plant expression except that the desired DNA region encoding a protein or portion thereof, which DNA coding region can readily be added to the vector by known methods. Generally, an intron of this invention is inserted into the 5' non-translated leader sequence.

Any promoter that is known or found to cause transcription of DNA in plant cells can be used in the present invention. The amount of enhancement of expression by use of the introns of this invention may vary from promoter to promoter as has been observed by use of other introns. See Callis et al., supra, and Mascanenkas et al., supra. Suitable promoters can be obtained from a variety of sources such as plants or plant DNA viruses and include, but are not necessarily limited to, promoters isolated from the caulimovirus group, such as the cauliflower mosaic virus 19S and 35S (CaMV19S and CaMV35S) transcript promoters or the figwort mosaic virus full-length transcript promoter (FMV35S). The FMV35S promoter causes a high level of uniform expression of a protein coding region coupled thereto in most plant tissues. Other useful promoters include the enhanced CaMV35S promoter (eCaMV35S) as

described by Kat et al. (1987) *Science* 236:1299–1302, and the small subunit promoter of ribulose 1,5-bisphosphate carboxylase oxygenase (RUBISCO).

Examples of other suitable promoters are rice actin promoter; cyclophilin promoter; ubiquitin promoter; ADH1 promoter, Callis et al., supra.; Class I patatin promoter, Bevan et al. (1986) *Nucleic Acids Res.* 14 (11), 4675–4638; ADP glucose pyrophosphorylase promoter;  $\beta$ -conglycinin promoter, Tiemey et al. (1987) *Planta* 172: 356–363; E8 promoter, Deikman et al. (1988) *Embo J.* 7 (11) 3315–3320; 2AII promoter, Pear et al. (1989) *Plant Mol. Biol.* 13: 639–651; acid chitinase promoter, Samac et al. (1990) *Plant Physiol.* 93: 907–914;

The promoter selected should be capable of causing sufficient expression of the desired protein alone, but especially when used with the HSP70 intron, to result in the production of an effective amount of the desired protein to cause the plant cells and plants regenerated therefrom to exhibit the properties which are phenotypically caused by the expressed protein. In particular, the enhanced CaMV35S promoter or the FMV35S promoter is useful in the present invention. The enhanced CaMV35S promoter causes sufficient levels of the protein mRNA sequence to be produced in plant cells.

The mRNA produced by the promoter contains a 5' non-translated leader sequence. This non-translated leader sequence can be derived from any suitable source and can be specifically modified to increase translation of the mRNA. The 5' non-translated region can be obtained from the promoter selected to express the gene, the native 5' leader sequence of the gene or coding region to be expressed, viral RNAs, suitable eucaryotic genes, or a synthetic gene sequence. The present invention is not limited to the construct presented in the following examples, wherein the non-translated region is derived from 45 nucleotides from the eCaMV35S promoter. The non-translated leader sequence can also be derived from an unrelated promoter or viral coding region as described.

The 3' non-translated region of the chimeric plant gene contains a polyadenylation signal that functions in plants to cause the addition of polyadenylated ribonucleotides to the 3' end of the mRNA. Examples of suitable 3' regions are the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the NOS gene, and plant genes such as the soybean storage protein genes and the small subunit promoter of the RUBISCO gene. An example of a preferred 3' region is that from the nopaline synthase gene as described in the examples below.

In order to determine that the isolated HSP70 intron sequence included the desired intron region and to demonstrate the effectiveness and utility of the isolated HSP70 intron, reporter genes were inserted into plant cassette vectors. The reporter genes chosen were the *E. coli*  $\beta$ -glucuronidase (GUS) coding sequence and the luciferase (LUX) coding sequence.

The chimeric gene of this invention may contain any structural gene encoding a protein to be expressed in plants. An example of a suitable protein for use in this invention is EPSP synthase (5-enolpyruvyl-3-phosphoshikimate synthase; EC:25.1.19) which is an enzyme involved in the shikimic acid pathway of plants. The shikimic acid pathway provides a precursor the the synthesis of aromatic amino acids essential to the plant. Specifically, EPSP synthase catalyzes the conversion of phosphoenol pyruvate and 3-phosphoshikimic acid to 5-enolpyruvyl-3-

phosphoshikimate acid. A herbicide containing N-phosphonomethylglycine inhibits the EPSP synthase enzyme and thereby inhibits the shikimic acid pathway of the plant. The term "glyphosate" is usually used to refer to the N-phosphonomethylglycine herbicide in its acidic or anionic forms. Novel EPSP synthase enzymes have been discovered that exhibit an increased tolerance to glyphosate containing herbicides. In particular, an EPSP synthase enzyme having a single glycine to alanine substitution in the highly conserved region having the sequence: -L-G-N-A-G-T-A- located between positions 80 and 120 in the mature wild-type EPSP synthase amino acid sequence has been shown to exhibit an increased tolerance to glyphosate and is described in the commonly assigned U.S. Pat. No. 4,971,908 entitled "Glyphosate-Tolerant 5-Enolpyruvyl-3-Phosphoshikimate Synthase," the teachings of which are hereby incorporated by reference hereto. Methods for transforming plants to exhibit glyphosate tolerance are discussed in the commonly assigned U.S. Pat. No. 4,940,835 entitled "Glyphosate-Resistant Plants," the disclosure of which is specifically incorporated herein by reference. A glyphosate-tolerant EPSP synthase plant gene encodes a polypeptide which contains a chloroplast transit peptide (CTP) which enables the EPSP synthase polypeptide (or an active portion thereto) to be transported into a chloroplast inside the plant cell. The EPSP synthase gene is transcribed into mRNA in the nucleus and the mRNA is translated into a precursor polypeptide (CTP/mature EPSP synthase) in the cytoplasm. The precursor polypeptide is transported into the chloroplast.

Another example of a suitable protein for use in this invention is glyphosate oxidoreductase (GOX) enzyme which is an enzyme which converts glyphosate to aminomethyl-phosphate and glyoxylate. By expressing the GOX enzyme in plants results in plants tolerant to glyphosate herbicide. The amino acid sequence of the GOX enzyme and modified genes encoding the GOX enzyme adapted for enhanced expression in plants are described in the commonly assigned patent application entitled "Glyphosate Tolerant Plants" having U.S. Ser. No. 07/717,370 filed Jun. 24, 1991, the teachings of which are hereby incorporated herein by reference.

Other examples of suitable proteins for use in this invention are *Bacillus thuringiensis* (*B.t.*) crystal toxin proteins which when expressed in plants protect the plants from insect infestation because the insect, upon eating the plant containing the *B.t.* toxin protein either dies or stops feeding. *B.t.* toxin proteins toxic to either Lepidopteran or Coleopteran insects may be used. Examples of particularly suitable DNA sequences encoding *B.t.* toxin protein are described in the commonly assigned patent application entitled "Synthetic Plant Genes and Method for Preparation," EP patent application 385,962 published Sep. 5, 1990, the teachings of which are hereby incorporated herein by reference.

Another example of an enzyme suitable for use in this invention is aminocyclopropane-1-carboxylic acid (ACC) oxidase which when expressed in plants delays fruit ripening by reducing the ethylene level in plant tissues.

Other examples of enzymes suitable for use in this invention are acetolactate synthase, RNase to impart male sterility, Mariani et al. (1990) *Nature* 347: 737–741, and wheat germ agglutinin.

Another example of an enzyme suitable for use in this invention is ADP glucose pyrophosphorylase which when expressed in plants enhances the starch content.

All oligonucleotides are synthesized by the method of Adams et al. (1983) *J. Amer. Chem. Soc.* 105, 661. The nucleotide bases adenine, thymine, uracil, cytosine and guanine are represented by the letters A, T, U, C and G, respectively.

This invention is suitable for any member of the monocotyledonous (monocot) plant family including, but not limited to, maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, yams, onion, banana, coconut, dates and hops. The present invention has particular applicability to the production of transgenic maize plants.

Any method suitable for transforming plant cells and regenerating transgenic plants may be used in the practice of this invention. Illustrative examples of methods suitable for regenerating transgenic plants are: corn (Fromm et al., 1990, *Bio/Technology* 8:833-839; and Gordon-Kamm et al., 1990, *The Plant Cell* 2:603-618); rice (Wang et al., 1988, *Plant Mol. Biol.* 11:433-439) and wheat (Vasil et al., 1991, *Bio/Technology* 8:743-747).

The production of fertile transgenic monocotyledonous plants involves several steps that together form the process. Generally, these steps comprise 1) culturing the desired monocot tissue to be transformed to obtain suitable starting material; 2) developing suitable DNA vectors and genes to be transferred into the monocot tissue; 3) inserting the desired DNA into the target tissue by a suitable method; 4) plant cells; 5) regenerating transgenic cells into fertile transgenic plants and producing progeny; and 6) analyzing the transgenic plants and their progeny for the presence of the inserted heterologous DNA or foreign gene.

A preferred method of the present invention utilizes embryogenic callus which is suitable for transformation and regeneration as the starting plant material. Embryogenic callus is defined as callus which is capable of being transformed and subsequently being regenerated into mature, fertile transgenic plants. The embryogenic callus preferably has a friable Type II callus phenotype that performs well in tissue culture. Embryogenic callus may be obtained using standard procedures known to those in the art (Armstrong, 1991, *Maize Genetic Newsletter* 65:92-93). Suitable maize embryogenic callus material may be obtained by isolating immature embryos from the maize plant 10 to 12 days after pollination. The immature embryos are then placed on solid culturing media to initiate callus growth. The immature embryos begin to proliferate as Type II callus after about one week and are thereafter suitable for use in the method of the present invention. Embryogenic callus suitable for use in the method of the present invention may be obtained from the initial callus formation on the immature embryos or may be from older established callus cultures up to 2 years in age. It is preferred, however, that younger callus cultures be used to enhance the recovery of fertile transgenic plants. Embryogenic callus that is between one week and six months of age is preferred and embryogenic callus between one week and four weeks of age is most preferred. The embryogenic callus of the present invention is considered "primary" callus in that it has never been processed through or maintained as a suspension culture. A suspension culture is defined as callus that has been broken up and placed in a liquid solution for a period of 1 to 9 months to establish a growing suspension culture. The embryogenic callus suitable for use in the present invention has never been through a suspension culture process or ever maintained as a suspension culture.

The preferred method of the present invention is applicable to any monocot embryogenic callus that is capable of regenerating into mature fertile transgenic plants and does

not depend on a particular genotype, inbred, hybrid or species of the monocot desired to be transformed. It is to be understood, however, that the efficiency of the process will probably vary depending on the culturability and transformability of the particular plant line being used. In the present invention, a preferred maize embryogenic callus may be obtained from an A188xB73 F<sub>1</sub> genotype hybrid line, or a derivative of this line, or an "Hi-II" genotype. Any genotype that can give rise to a friable Type II callus material is suitable and will be useful in the method of the present invention. The embryogenic callus may be initiated and maintained in any suitable tissue culture media that will promote the growth of callus of the desired phenotype. Suitable tissue culture media are known to those skilled in the art of plant genetic engineering. The A188xB73 F<sub>1</sub> hybrid line and Hi-II line have been successfully initiated, maintained and regenerated in the tissue culture media described in Table 1.

TABLE 1

N6 1-100-25 (1 L)  
4.0 grams/L Chu (N<sub>6</sub>) Basal Salts (Sigma C-1416)  
1.0 ml/L Eriksson's Vitamin Mix (1000xstock made from Sigma E-1511 Powder)  
1.25 ml/L 0.4 mg/ml thiamine HCl  
20 g/L Sucrose  
1 ml/L 2, 4 D (1 MG/ML) (2, 4 D=2, 4, dichlorophenoxyacetic acid)  
2.88 g/L L-proline  
0.1 g/L Vitamin Free Casamino Acids (from Difco; Bacto Vitamin Assay Casamino Acids, Catalog#0288-01-2).  
Adjust pH to 5.8, and add 2 g/L Gelrite or Phytagel, autoclave for 30 minutes, and pour into 25x100 mm petri dishes in sterile hood.  
N6 1-0-25 same as N6 1-100-25 except that no Casamino Acids are used.  
N6 2-100-25 same as N6 1-100-25 above, except that 2 ml/L 2,4 D (1 mg/ml) is used.  
N6 2-0-0 same as N6 1-100-25 above, except 2 ml/L 2,4 D (1 mg/ml) and no Vitamin Free Casamino Acids and no L-proline.  
N6 6% 0 D same as N6 2-0-0 above, except 60 g/L sucrose 0 ml/L 2,4 D, and 0 L-proline is used.  
MS 0.1 D 4.3 g/L MS salts (Sigma), 20 g/L sucrose, 100 mg/L myo-inositol, 1.3 mg/L nicotinic acid, 0.25 mg/L each of thiamine-HCL, pyridoxine and calcium pantothenate, 0.1 ml/L of 2, 4 D (1 mg/ml), 10-7M Absciscic Acid (ABA).  
MS 0 D same as MS 0.1 D above, except no 2, 4 D and no ABA.

Once the desired embryogenic callus culture has been obtained, transformation of the tissue is possible. A foreign gene or genes of interest may be transferred to the embryogenic callus. Generally, the DNA inserted into the embryogenic callus is referred to as heterologous DNA. The heterologous DNA may contain one or more foreign genes which may or may not be normally present in the particular monocotyledonous plant being transformed. A foreign gene is typically a chimeric or recombinant gene construct comprising a sequence of DNA which may or may not be normally present in the genome of the particular monocot being transformed. The heterologous DNA generally contains a foreign gene which comprises the necessary elements for expression of a desired polypeptide in the particular plant. Heterologous DNA suitable for transformation into a

monocotyledonous plant typically contains foreign genes coding for a polypeptide which confers a desired trait or characteristic to the plant being transformed and screenable and selectable markers for determining whether the plant material has been transformed. A typical foreign gene capable of being expressed in a monocot contains a promoter which is capable of functioning in the monocot plant, an intron, a structural DNA coding sequence encoding a desired polypeptide and a polyadenylation site region recognized in monocotyledonous plants. A transgene is a gene or DNA sequence that has been transferred into a plant or plant cell. The details of construction of heterologous DNA vectors and/or foreign genes suitable for expression in monocots is known to those skilled in the art of plant genetic engineering. The heterologous DNA to be transferred to the monocot embryogenic callus may be contained on a single plasmid vector or may be on different plasmids.

The heterologous DNA to be used in transforming the embryogenic callus in the method of the present invention preferably includes a selectable marker gene which allows transformed cells to grow in the presence of a metabolic inhibitor that slows the growth of non-transformed cells. This growth advantage of the transgenic cells allows them to be distinguished, over time, from the slower growing or non-growing cells. Alternatively, or in conjunction with a selectable marker, a visual screenable marker such as the *E. coli*  $\beta$ -glucuronidase gene or firefly luciferase gene (deWet et al., 1987, *Mol. Cell Biol.* 7:725-737) also facilitates the recovery of transgenic cells.

Preferred selectable marker genes for use in the method of the present invention include a mutant acetolactate synthase gene or cDNA which confers tolerance to sulfonyleurea herbicides such as chlorsulfuron, the NPTII gene for resistance to the antibiotic kanamycin or G418 or a bar gene for resistance to phosphinothricin or bialaphos.

The foreign gene selected for insertion into the monocot embryogenic callus can be any foreign gene which would be useful if expressed in a monocot. Particularly useful foreign genes to be expressed in monocots include genes which confer tolerance to herbicides, tolerance to insects, tolerance to viruses, and genes which provide improved or new characteristics which effect the nutritional value or processing capabilities or qualities of the plant. Examples of suitable agronomically useful genes include the insecticidal gene from *Bacillus thuringiensis* for conferring insect resistance and the 5'-enolpyruvyl-3'-phosphoshikimate synthase (EPSPS) gene and any variant thereof for conferring tolerance to glyphosate herbicides. As is readily understood by those skilled in the art, many other agronomically important genes conferring desirable traits can be introduced into the embryogenic callus in conjunction with the method of the present invention. One practical benefit of the technology of the present invention is the production of transgenic monocotyledonous plants that have improved agronomic traits.

Once the transformation vectors containing the desired heterologous DNA have been prepared, the DNA may be transferred to the monocot embryogenic callus through use of the microprojectile bombardment process which is also referred to as particle gun technology or the Biolistics process. The heterologous DNA to be transferred is initially coated onto a suitable microprojectile by any of several methods known to those skilled in the plant genetic engineering art. The microprojectiles are accelerated into the target embryogenic callus by a microprojectile gun device. The design of the accelerating device or gun is not critical so long as it can perform the acceleration function. The accelerated microprojectiles impact upon the prepared

embryogenic callus to perform the gene transfer. When the microprojectile bombardment process is utilized, the DNA vector used to transfer the desired genes to the embryogenic callus is typically prepared as a plasmid vector and is coated onto tungsten or gold microprojectiles.

While any particle gun device may be used, the Biolistics PDS 1000 microprojectile gun device was used in the present invention. This device had a stopping plate configuration similar to commercially available stopping plates except that the lexan disk is  $\frac{3}{8}$ " thick with a  $\frac{3}{32}$ " diameter hole through the disk center. The hole is enlarged at the upper surface to  $\frac{7}{16}$ " and this tapers in a countersunk arrangement to a depth of  $\frac{1}{4}$ " at which point it narrows to the  $\frac{3}{32}$ " diameter hole which does not have a taper for the remaining  $\frac{1}{18}$ " thickness. The embryogenic target tissue is set at level 4 of this device which is one level from the bottom. The callus tissue sample was subjected to 1-3 shots. A shielding metal screen with 100 $\mu$  openings is typically used on the shelf position immediately below the stopping plate. The process is performed under a suitable vacuum.

After the embryogenic calli have been bombarded with the desired heterologous DNA vector, the bombarded cells are grown for several days in non-selective culturing media and then placed on a selective media which inhibits the growth of the non-transformed cells, but allows transgenic cells to continue to grow. In about 8 weeks, the continued growth of the transgenic callus cells is apparent as a large growing calli and can be recovered and individually propagated. The transgenic embryogenic callus may then be regenerated into whole, mature transgenic plants pursuant to protocols for regenerating non-transformed embryogenic callus. Generally, when regenerated plants reach the three-leaf stage and have a well developed root system, they can be transferred to soil and hardened off in a growth chamber for two weeks before transfer to a greenhouse. The transformed embryogenic callus of the present invention respond well to regeneration procedures which work for non-transgenic callus.

Regenerated plants may subsequently be moved to a greenhouse and treated as normal plants for pollination and seed set. The confirmation of the transgenic nature of the callus and regenerated plants may be performed by PCR analysis, antibiotic or herbicide resistance, enzymatic analysis and/or Southern blots to verify transformation. Progeny of the regenerated plants may be obtained and analyzed to verify the heritability of the transgenes. This illustrates the stable transformation and inheritance of the transgenes in the  $R_1$  plant.

The following examples are provided to illustrate the method of the present invention and should not be interpreted in any way to limit the scope of the invention. Those skilled in the art will recognize that various modifications can be made to the methods described herein while not departing from the spirit and scope of the present invention.

#### DESCRIPTION OF PREFERRED EMBODIMENTS

##### Example 1

##### Synthesis of HSP70 Intron by Polymerase Chain Reaction

The HSP70 intron was synthesized using the polymerase chain reaction from a genomic clone containing a maize HSP70 gene (pMON9502: Rochester et al., 1986, *Embo J.*, 5:451-458).

Two different oligonucleotide primers were used in the PCR reaction. The first primer consists of nucleotides 1-26 of SEQ ID NO:1 and contains a BglIII site for cloning, ten

nucleotides of flanking HSP70 exon 1 sequence, and ten bases of the intron sequence. The second primer is the reverse complement of bases 791 through 816 of SEQ ID NO:1 and contains 10 bp of intron sequence, 11 nucleotides of flanking 3' HSP70 exon sequence, and an NcoI site for cloning.

The "HSP70 intron," bases 7–812, contains the entire intron from a maize HSP70 gene (bases 17–799) plus 10 nucleotides from HSP70 exon 1 (bases 7–16) and 11 bases from HSP70 exon 2 (bases 800–812). Bases 1–6 and 813–816 include restriction sites used in cloning. Base 802 was a G in the native HSP70 exon, but has been replaced by an A for maximum enhancement of gene expression.

PCR was carried out in 100 µl reactions which contained 10 ng pMON9502 DNA, 40 pmole each of SEQ7 and SEQ20, 10 mM Tris-HCL (pH8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01%(w/v) gelatin, 20 nmole of each dNTP, and 2.5 units Amplitaq DNA Polymerase (Perkin Elmer Cetus). Twenty eight cycles were run (denaturation 1 minute at 94° C., annealed 2 minutes at 50° C., and elongated 3 minutes at 72° C. per cycle).

The PCR reaction products were purified by phenol:chloroform (1:1) extraction, followed by digestion with BglII and NcoI. The 0.8 kb HSP70 intron fragment was isolated by gel electrophoresis followed by purification over an Elutip-D column (Schlesser & Schuell). The HSP70 intron sequences were verified by the Sanger dideoxy DNA sequencing method. The sequence of the HSP70 intron is designated SEQ ID NO:1 and is shown in FIG. 1. The 0.8 kb HSP70 intron fragment was then cloned into the BglII and NcoI sites within the 5' untranslated leader region of pMON8677 to form pMON19433 as described below.

#### Example 2

Effect of HSP70 Intron on Corn Gene Expression in Transient Assays

A. Preparation of pMON8677, pMON8678, pMON19433, pMON19425, pMON19400, and pMON19437

pMON8677 (FIG. 4) was constructed using well characterized genetic elements. The 0.65 kb cauliflower mosaic virus (CaMV) 35S RNA promoter (e35S) containing a duplication of the –90 to –300 region (Kay et al., 1987, *Science* 236:1299–1302), the 1.9 kb coding sequences from the *E. coli* β-glucuronidase (GUS) gene (Jefferson et al., 1986, *PNAS* 83:8447–8451) and a 0.25 kb fragment containing the 3' polyadenylation sequences from the nopaline synthase (NOS) gene (Fraley et al., 1983, *Proc. Natl. Acad. Sci.* 80:4803–4807) were each inserted into pUC119 (Yanisch-Perron et al., 1985, *Gene* 33:103–119) to form the plant gene expression vector pMON8677.

pMON8678 (FIG. 5) was formed by inserting a 0.6 kb fragment containing the first intron from the ADH1 gene of maize (Callis et al., 1987, *Genes and Dev.* 1:1183–1200) into pMON8677 as described in Vasil et al. (1991) *Bio/Technology* 9:743–747. The monocot expression region in pMON8678 is identical to pMON8677 except that it contains the ADH1 intron fragment in the 5' untranslated leader.

pMON19433 (FIG. 9) was constructed by cloning the BglII-NcoI digested PCR fragment containing the maize HSP70 intron sequences into the NcoI-BglII sites in pMON8677 to produce a monocot expression vector equivalent to pMON8677 except that it contains the maize HSP70 intron fragment in the 5' untranslated leader.

pMON19425 (FIG. 6) was constructed by inserting the 0.65 kb cauliflower mosaic virus (CaMV) 35S RNA promoter (e35S) containing a duplication of the –90 to –300 region, the 1.8 kb fragment of the firefly luciferase (LUX) gene (Ow et al., 1986, *Science* 234:856–859; DeWet et al.,

1987, *Mol. Cell Biol.* 7:725–737), and the 0.25 kb fragment containing the NOS polyadenylation sequences into pUC119 (Yanisch-Perron et al., 1985, supra).

pMON19400 (FIG. 18) was formed by replacing the GUS coding sequence in pMON8678 with the 1.8 kb fragment of the LUX gene. The monocot expression region in pMON19400 is identical to pMON19425 except that it contains the ADH1 intron fragment in the 5' untranslated leader.

pMON19437 (FIG. 10) was constructed by cloning HSP70 intron sequence from pMON19433 as a 0.8 bp NcoI-BglII fragment into the NcoI-BglII sites in pMON19425 to produce a monocot expression vector equivalent to pMON19425 except that it contains the maize HSP70 intron fragment in the 5' untranslated leader.

#### B. Analysis of Gene Expression Using Transient Assays

Two transient gene expression systems were used to evaluate expression from the HSP70 intron and ADH1 intron vectors in corn cells. Two corn cell lines were transformed shooting corn cells or tissues by high velocity projectiles coated with the indicated plasmid DNA. One cell line was Black Mexican Sweet (BMS) corn, a nonregenerable corn callus suspension cells. The other cell line was BC17 corn used as tissue from corn leaves obtained from 4 week old plants from the innermost leaves at the nodes around the tassel primordia.

Plasmid DNAs were prepared by using standard alkaline lysis followed by CsCl gradient purification (Maniatis et al., 1982, *Molecular Cloning: A Laboratory Manual*, CSH Labs). Plasmid DNA was precipitated onto tungsten M10 particles by adding 25 µl of particles (25 mg/ml in 50% glycerol), 3 µl experimental plasmid DNA (1 µg/µl), 2 µl internal control plasmid DNA (1 µg/µl), 25 µl 1M calcium chloride, and 10 µl 0.1M spermidine, and vortexing briefly. The particles were allowed to settle for 20 minutes, after which 25 µl of supernatant was removed and discarded. Two independent particle preparations were done for each vector evaluated.

The particle preparations were then bombarded into the tissue/cells as follows. Each sample of DNA-tungsten was sonicated briefly and 2.5 µl was bombarded into the tissue/cells contained on one plate using a PDS-1000 (DuPont) Biolistics particle gun. Three plates of tissue/cells were bombarded from each particle preparation.

The tissue/cells were harvested after a 24–48 hours incubation (25° C., dark). The cells/tissues from the three bombarded plates from each particle preparation were combined, frozen with liquid nitrogen, and ground to a fine white powder with a mortar and pestle. Each sample was thawed on ice in 1 ml of GUS extraction buffer (GEB: 0.1M KPO<sub>4</sub> pH7.8, 1 mM EDTA, 10 mM DTT, 0.8 mM PMSF, and 5% glycerol). The samples were then vortexed and centrifuged at 8K for 15 minutes at 5° C., and the supernatant was transferred to a fresh tube. When enzyme assays were not performed immediately, the samples were frozen on dry ice and stored at –80° C.

Transient β-glucuronidase gene expression was quantitated using a fluorometric assay (Jefferson et al., 1987, *Embo. J.* 6:3901–3907). Fifty µl crude extract was assayed in one ml GEB containing 2 mM 4-methyl umbelliferyl glucuronide. At 0, 10, 20, and 30 minute timepoints, 100 µl aliquots were removed and the reaction terminated by addition to 2 ml 0.2M Na<sub>2</sub>CO<sub>3</sub>. Fluorescence from each sample was then determined using a Hoescht DNA Fluorometer (model TKO 100). GUS activity is expressed as the slope of fluorescence versus reaction time.

Quantitative luciferase assays were performed as follows. 50 µl of extract was added to a cuvette containing 0.2 mls of

25 mM Tricine pH7.8, 15 mM MgCl<sub>2</sub>, 5 mM ATP, and 0.5 mg/ml BSA. The 0.5 mM luciferin substrate was automatically dispensed by the luminometer (Berthold Bioluminat LB9500C) and the peak luminescence measured during a 10 second count at 25° C. Three to ten reactions were run per sample. LUX activity is expressed as the mean light units per ul of extract.

All vectors tested were co-bombarded with internal control vectors which encoded proteins whose enzymatic activities were distinct from those of the vectors being evaluated. For example, in the experiments in which LUX vectors being evaluated, pMON8678 (GUS) was used as the internal control vector, and when GUS vectors being tested pMON19400 (LUX) was used as the internal control vector. To correct for any variability in the procedure the results were then expressed as a ratio of the experimental reporter gene expression to the internal control reporter gene expression. The results are summarized in Table 2.

As shown in Table 2A, the HSP70 intron vectors gave significantly increased gene expression in BMS suspension cells when compared to vectors containing no intron (40 fold increase) or the ADH1 intron (4 fold increase) vectors. This effect was observed using either GUS or or LUX as the reporter gene. Table 2B shows that this effect is not limited to the BMS cell system. In the leaf transient gene expression assays, the HSP70 intron vector showed an 8.7 times GUS expression level over the control containing no intron, whereas, the ADH1 intron showed only a 1.6 times GUS expression level over the control containing no intron.

TABLE 2

Effects of Introns on Gene Expression in Transient Assays		
Intron	Relative GUS (vector)	Relative LUX (vector)
A. Effect of introns on transient gene expression in BMS cells.		
no intron	1X (pMON8677)	1X (pMON19425)
ADH1	4X (pMON8678)	4X (pMON19400)
HSP70	40X (pMON19433)	40X (pMON19437)
B. Effect of introns on transient gene expression in maize leaf tissue.		
no intron	1X (pMON8677)	
ADH1	1.6X (pMON8678)	
HSP70	8.7X (pMON19433)	

### Example 3

Effect of HSP70 Intron on Gene Expression in Stably Transformed Nonregenerable Corn Cultures

#### A. Production of stably transformed BMS cell lines

Black Mexican Sweet corn suspension cells were transformed by particle gun bombardment essentially as described above. Plasmid DNA for bombardment was prepared and precipitated onto tungsten M10 particles by adding 12.5 ul of particles (25 mg/ml in 50% glycerol), 2.5 ul plasmid DNA (1 ug/ul), 12.5 uL 1M calcium chloride, and 5 uL 0.1M spermidine, and vortexing briefly. The particles were allowed to settle for 20 minutes, after which 12.5 ul of supernatant was removed and discarded. Each sample of DNA-tungsten was sonicated briefly and 2.5 ul was bombarded into the embryogenic cultures using a PDS-1000 biolistics particle gun (DuPont). EC9 (FIG. 19), a plasmid containing an acetolactate synthase gene, was included for use in chlorsulfuron selection for transformed control cells. A second plasmid containing the test construct was co-precipitated with EC9. BMS cells were plated on filters and bombarded using a PDS-1000 (DuPont) particle gun.

After bombardment, the cells were transferred to MS liquid medium for 1 day and then plated onto solid medium containing 20 ppb chlorsulfuron. After approximately 4 weeks, chlorsulfuron resistant calli were selected and grown up for analysis of gene expression.

#### B. Effect of the HSP70 Intron on GUS Expression

Plasmids containing the GUS gene and no intron (pMON8677), ADH1 intron (pMON8678), or HSP70 intron (pMON19433) were bombarded into BMS cells and stably transformed lines were produced as described above. Chlorsulfuron resistant lines were selected and then scored for GUS expression by histochemical staining (Jefferson et al., 1987, *Embo. J.* 6:3901-3907). As shown in Table 3A, the transformations with the HSP70 intron vector showed a significantly higher proportion of co-expression of the unselected GUS marker than did the transformation with either the vector containing the ADH1 intron or no intron. Since more chlorsulfuron resistant calli were above the threshold of detection histochemical GUS staining, it is likely that the HSP70 intron vectors express at higher levels than the ADH1 or no intron vectors. To confirm this, GUS activity was quantitated in extracts from ten independent GUS positive transformants from each vector (for pMON8677, the one GUS positive callus was assayed; nine others were chosen randomly). The data from these assays is shown in Table 3B. These results indicate that the HSP70 intron enhances GUS expression in stably transformed cell lines to an even greater extent than was observed in transient gene expression analyses. The mean level of GUS expression observed with the lines containing the HSP70 intron vector was approximately 80 fold over that observed in lines containing the ADH1 intron vector. The best of the ten HSP70 lines expresses over 100 fold more GUS than the best ADH1 line and approximately 800 fold over the best line without an intron.

TABLE 3

Effect of Introns on GUS Expression in Stable Transformants			
A. GUS expressing BMS calli - number and percentage.			
Class*	pMON8677	pMON8678	pMON19433
	No Intron	ADH1 intron	HSP70 intron
-	79 (99%)	48 (67%)	28 (47%)
+	0 (0%)	14 (19%)	2 (3%)
++	1 (1%)	9 (13%)	7 (12%)
+++	0 (0%)	1 (1%)	22 (37%)
	80	72	59
B. Levels of GUS expression in BMS calli.			
Vector	Intron	Range**	Mean**
pMON8677	none	0-38	N.D.
pMON8678	ADH1	28-219	95 + 75
pMON19433	HSP70	1594-29,629	7319 ± 9016

\* - no cells show expression

+ a few cells show GUS expression

++ some cells show GUS expression

+++ all cells show strong GUS expression

\*\* (pmol/min/mg)

### Example 4

Effect of HSP70 Intron on *B.t.k.* Expression in Stably Transformed BMS Cell Lines

We have similarly examined the effect of the HSP70 intron on expression of the commercially important *B.t.k.* gene. Two plasmids were constructed that only differed by

the intron they contained: pMON10920 (e35S/ADH1/*B.t.k.*/NOS) and pMON10921 (e35S/HSP70/*B.t.k.*/NOS). Each contained a 3.6 kb fully synthetic gene encoding the *Bacillus thuringiensis* kurstaki (*B.t.k.*) insect control protein described by Adang et al. (1985) *Gene* 36: 289–300. Expression of this gene in plants results in insect resistance. pMON10920 (FIG. 20) was constructed by inserting the 3.6 kb NcoI/EcoRI fragment containing the *B.t.k.* into pMON8678 (FIG. 5), replacing the 1.9 kb GUS fragment. pMON10921 (FIG. 11) was constructed similarly, except that the 3.6 kb NcoI/EcoRI fragment containing the *B.t.k.* coding sequence was inserted into pMON19433 (FIG. 9).

BMS lines were co-transformed with each of these plasmids and EC9 (ALS) as described in Example 3A. Approximately thirty independent chlorsulfuron resistant lines were generated in each transformation. These calli were tested for Tobacco Hornworm (THW) toxicity, and the insect resistant lines were assayed further. The amount of *B.t.k.* protein in soluble extracts from each THW resistant callus was measured by ELISA and expressed as a percentage of total protein. Of the 11 insect positive lines containing the ADH1 intron vector (pMON10920), only one line contained enough *B.t.k.* protein to be detected in the ELISA assay. The amount was  $0.4 \times 10^{-5}\%$ . Twenty of the 29 THW resistant lines containing the HSP70 intron vector (pMON10921) produced enough protein for detection by ELISA. The average amount was  $5.1 \times 10^{-5}\%$  with a range of  $<0.01$ – $10.5 \times 10^{-5}\%$ . When the mean *B.t.k.* protein levels are compared, the HSP70 intron vector increases expression 12 fold over the ADH1 intron vector.

#### Example 5

Effect of HSP70 Intron on GOX Expression in BMS Transformants

pMON19632 and pMON 19643 were constructed to examine the effects of introns on GOX expression. Both vectors contain a gene fusion composed of the N-terminal 0.26 Kb chloroplast transit peptide sequence derived from the *Arabidopsis thaliana* SSU 1a gene (SSU CTP) (Timko et al., 1988, *The Impact of Chemistry on Biotechnology*, ACS Books, 279–295) and the C-terminal 1.3 Kb synthetic GOX gene sequence. The GOX gene encodes the enzyme glyphosate oxidoreductase which catalyzes the conversion of glyphosate to herbicidally inactive products, aminomethylphosphonate and glyoxylate. Plant expression of the gene fusion produces a pre-protein which is rapidly imported into chloroplasts where the CTP is cleaved and degraded releasing the mature GOX protein (della-Cioppa et al., 1986, *Proc. Natl. Acad. Sci. USA* 83: 6873–6877).

pMON19632 (FIG. 22) was constructed in the same manner as pMON8678 by inserting the SSU-CTP—GOX fusion as a 1.6 kb BglII-EcoRI fragment between the ADH1 intron and NOS polyadenylation sequences. Thus, pMON19632 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, ADH1 intron, SSU-CTP—GOX coding sequence, and nopaline synthase polyadenylation region in a pUC backbone containing an  $\beta$ -lactamase gene for ampicillin selection in bacteria.

A cassette vector pMON19470 was constructed for cloning coding sequences such as GOX adjacent to the HSP70 intron (FIG. 7). A receptor plasmid pMON19453 was made by inserting annealed synthetic oligonucleotides containing the sites KpnI/NotI/HincII/HindIII/BglII/DraI/XbaI/NcoI/BamHI/EcoRI/EcoRV/XmaI/NotI/SacI into PBSKS+ (Stratagene) which had been digested with KpnI and SacI. The nopaline synthase (NOS) polyadenylation region (Fraleley et al., 1983, *Proc. Natl. Acad. Sci.* 80:4803–4807) was inserted by digesting pMON8678 (FIG. 5) with BamHI,

followed filling Klenow Polymerase to create blunt ends, and digesting with EcoRI. The 0.25 kb NOS fragment was inserted into the polylinker of pMON19453 at the EcoRV/EcoRI sites to form pMON19459. pMON19457 was constructed by inserting a 0.65 kb fragment containing the CaMV E35S promoter (Kay et al., 1987, *Science* 236:1299–1302) into the HindIII/BglII sites in pMON19459. pMON19433 was linearized with NcoI, blunt-ended with mung bean nuclease, and Xba linkers were added. The HSP70 intron fragment was then removed by digestion with BglII and inserted into the XbaI/BglII sites in pMON19457 to form pMON19458. Synthetic linkers to change the order of the restriction sites were then inserted into pMON19458 to form pMON19467. The NotI expression cassette was removed from pMON19467 and inserted into a pUC-like vector pMON10081 which contains the NPTII sequences from pKC7 (Rao and Rogers, 1978, *Gene* 3:247) to form pMON19470 (FIG. 8). Thus, pMON19470 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, polylinker for cloning coding sequences, and NOS polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

pMON19643 (FIG. 17) was constructed by inserting the SSU-CTP—GOX fusion coding sequences into pMON19470 as a 1.6 kb BglII/EcoRI fragment into BamHI-EcoRI digested pMON19470 (FIG. 8). Thus, pMON19643 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, SSU-CTP—GOX coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

BMS suspension cells were bombarded with pMON19632 or pMON19643 as described in Example 3A. Plasmid EC9 was included in each bombardment so that the transformed BMS cells could be selected on chlorsulfuron. The chlorsulfuron resistant calli were transferred to 5 mM glyphosate medium and moved to fresh 5 mM glyphosate medium after two weeks. After two weeks, the percentage of the calli that survived on the glyphosate medium were scored.

The results are shown in Table 4. The ADH1 intron vector (pMON19632) gave little or no glyphosate resistant calli. The HSP70 intron vector (pMON19643) showed over 40% of the chlorsulfuron resistant calli were also resistant to glyphosate. The levels of GOX protein accumulation in the chlorsulfuron resistant lines were measured by Western blot analysis. As shown in Table 3, the HSP70 intron vector gave demonstrably higher levels of GOX expression than the ADH1 intron vector.

TABLE 4

Effect of Introns on GOX Gene Expression in BMS Transformants			
Vector	Intron	% glp resistant	% GOX protein
pMON19632	ADH1	2%	(0.02–0.04%)
pMON19643	HSP70	42%	(0.05–0.5%)

#### Example 6

Effect of HSP70 Intron on EPSP Synthase and Glyphosate Selection

Two vectors, pMON8631 and pMON19640, were constructed to compare the effects of the ADH1 and HSP70 intron on the expression of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. pMON8631 (FIG. 23)

was constructed similarly to pMON8678 (FIG. 5), except that a 1.75 kb fragment containing the maize EPSPS coding sequence with two mutations that confer tolerance the herbicide glyphosate (Gly101>Ala and Gly163>Asp of mature peptide) was inserted between the ADH1 intron and the NOS polyadenylation sequences. Thus, pMON8631 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, ADH1 intron, EPSPS coding sequence, and nopaline synthase polyadenylation region in a pUC backbone containing a 13-lactamase gene for ampicillin selection in bacteria.

To form pMON19640 (FIG. 12), the 1.75 kb XbaI-EcoRI fragment from pMON8631 was inserted into the corresponding restriction sites in pMON19470 (FIG. 8). Thus, pMON19640 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, EPSPS coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

Stably transformed BMS lines were produced by direct selection on glyphosate containing medium. Cells were bombarded with either pMON8631 or pMON19640 as in described in Example 3A. After bombardment, the cells were resuspended in MS medium without selection for one day. Glyphosate was then added to the liquid medium to a final concentration of 5 mM, and the cultures incubated for four days. Five days post-bombardment, the cells were embedded in agarose containing 5 mM glyphosate. Approximately 6 weeks after embedding, the number of glyphosate resistant calli were scored. pMON8631 (ADH1 intron) produced 59 glyphosate resistant calli, while pMON19640 (HSP70 intron) produced 117 glyphosate resistant calli, a two fold increase. Although the levels of EPSPS expression in these calli was not quantitated, it is likely that the HSP70 intron vector expresses more EPSPS which in turn results in more transformation events that produce enough EPSPS to overcome the toxic effects of the glyphosate in the medium, thus giving a higher frequency of recovery of glyphosate resistant calli.

#### Example 7

##### Expression of Other Coding Sequences Using HSP70 Intron Vectors Encoding Insecticidal Proteins

pMON19484 (FIG. 13) containing a synthetic gene encoding the *Bacillus thuringiensis* var. tenebrionis (*B.t.t.*) insecticidal protein (McPherson et al., 1988, *Bio/Technology* 6: 61-66) was constructed by inserting the 1.8 kb *B.t.t.* gene on a BglII fragment into the BamHI site in pMON19470 (FIG. 8). Thus, pMON19484 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, *B.t.t.* coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

Stably transformed BMS calli were produced using particle gun bombardment to introduce pMON19484 as described in Example 3A. pMON19484 was bombarded in combination with EC9 (FIG. 19) into BMS cells. Resistant calli were selected on 20 ppb chlorsulfuron. The resistant calli were then assayed for expression of the *B.t.t.* gene.

Chlorsulfuron resistant calli bombarded with pMON19484 were screened for expression of the *B.t.t.* protein utilizing a Colorado Potato Beetle (CPB) feeding assay. CPB larvae were applied to BMS callus which had been blotted slightly to remove excess moisture. Five larvae were allowed to feed on callus representing each chlorsulfuron resistant line. The level of insect mortality and/or stunting was assessed five days later. Forty calli were assayed. Eight calli (20%) showed insecticidal activity, 11

calli (28%) caused stunting, 6 calli (15%) caused small amounts of stunting, and 15 calli (38%) had no effect on the CPB insects.

The calli that showed the greatest insecticidal/stunting effects were analyzed further by Western blot analysis. BMS calli were dried on a Whatman filter and then extracted directly in SDS-PAGE buffer (Laemmli, 1970, *Nature* 227: 680-685). Levels of total protein were determined (Biorad) and 40-50 ug protein loaded on a 12% SDS-PAGE gel. *E. coli*-produced *B.t.t.* protein was also loaded as quantitation standards. After gel electrophoresis, proteins were electrophoretically transferred from the gel to membranes (Towbin et al., 1979, *PNAS* 76:4350-4354). The membranes were then incubated with an anti-*B.t.t.* antibody, followed by detection using a chemiluminescent (Amersham) detection system.

Seven lines were examined. One line showed high levels of protein expression (0.02% total protein), four lines showed moderate *B.t.t.* protein levels (0.001%), and two lines did not produce enough *B.t.t.* protein for detection by Western blot.

pMON19486 (FIG. 14) contains a synthetic gene encoding the *Bacillus thuringiensis* kurstaki CryIIA gene. The amino acid sequence of this gene (1.9 kb) is identical to the gene referred to as the CryB1 in Widner et al. (1989) *J. Bacteriol.* 171:965-974. It has insecticidal activity against both lepidopteran and dipteran insects. pMON19486 was constructed by inserting the 1.9 kb CryIIA coding sequence on a BglII fragment into the BamHI site in pMON19470 (FIG. 8). Thus, pMON19486 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, CryIIA coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

Stably transformed BMS calli were produced using particle gun bombardment to introduce pMON19486 as described in Example 3A. pMON19484 was bombarded in combination with EC9 (FIG. 19) into BMS cells. Resistant calli were selected on 20 ppb chlorsulfuron. The resistant calli were then assayed for expression of the CryIIA gene.

Expression of the *B.t.k.* CryIIA protein in the chlorsulfuron resistant calli bombarded with pMON19486 was initially detected by insecticidal activity in a feeding assay with the sensitive Tobacco Hornworm (THW). Calli with CryIIA expression high enough to kill the THW insects were bulked up and assayed in European Corn Borer (ECB) and Fall Army Worm (FAW) insect feeding assays. Sixteen ECB or 12 FW insects were pre-weighed and then reared on the BMS calli for 7 days. The number of survivors were scored to determine the degree of mortality. The amount of stunting was measured by determining the average weight gain of the surviving insects relative to controls. The data are shown in Table 5.

Calli with insecticidal activity were also assayed for accumulation of the CryIIA protein by Western blot analysis as described above. The amount of CryIIA protein was quantitated relative to *E. coli* produced standards on the same blot. As shown in Table 5, six of the seven insecticidal lines demonstrated sufficient expression of the CryIIA protein to detect by the less sensitive Western blot. The CryIIA expression ranged from 0.004 to 0.15%, with an average of 0.007%, of total cellular protein.



TABLE 5

Expression of CryIIA in Stable BMS Transformants					
Line	# survivors/initial		mean weight gain per surviving insect (mg)		CryIIA protein (%)
	ECB	FAW	ECB	FAW	
control	10/16	10/12	3.0	3.9	0
12-9	0/16	10/12	all dead	0.5	0.004
3-20	2/16	11/12	0.8	1.5	0.004
11-31	1/16	10/12	3.7	1.2	0.015
3-4	0/16	11/12	all dead	0.6	0.013
3-10	1/16	12/12	3.5	0.8	0
3-38	0/16	11/12	all dead	0.4	0.0025
3-34	0/16	11/12	all dead	0.5	0.0025

## Example 8

pMON18103 (FIG. 16) contains a gene fusion composed of the N-terminal 0.26 Kb chloroplast transit peptide sequence derived from the Arabidopsis thaliana SSU 1a gene (SSU-CTP) (Timko et al., 1988, *The Impact of Chemistry on Biotechnology*, ACS Books, 279-295) and the *E. coli* ADP-glucose pyrophosphorylase mutant gene glgC16 (Leung et al., 1986, *J. Bacteriol.* 167: 82-88). Expression of the SSU-CTP/glgC16 fusion results in increased starch accumulation in plant cells. pMON18103 was constructed by inserting the SSU-CTP/glgC16 coding sequence on a 1.6 kb XbaI fragment into the XbaI site in pMON19467 (see FIG. 23). Thus, pMON19486 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, SSU-CTP/glgC16 coding sequences, and nopaline synthase polyadenylation region in a pUC backbone containing a  $\beta$ -lactamase gene for ampicillin selection in bacteria.

Stably transformed BMS calli were produced using particle gun bombardment to introduce pMON18103 as described in Example 3A. pMON19103 was bombarded in combination with EC9 (FIG. 19) into BMS cells. Resistant calli were selected on 20 ppb chlorsulfuron. The resistant calli were then assayed for expression of the glgC16 gene.

Chlorsulfuron resistant BMS lines that had been bombarded with pMON18103 were assayed for starch accumulation using I<sub>2</sub>/IKI staining (Coe et al., 1988, in *Corn and Corn Improvement*, eds. GF Sprague and JW Dudley. AGS Inc., Madison, Wisc. pp. 81-258). Eight of 67 lines showed increased levels of starch staining relative to control calli. Western blot analyses were performed on these lines as described above. All lines showed ADP-GPP expression, with levels from 0.02-0.1% of total protein relative to quantitation standards using *E. coli*-produced ADP-GPP protein.

## Example 9

pMON18131 (FIG. 15) contains the ACC deaminase gene from *Pseudomonas*. The ACC deaminase enzyme converts 1-aminocyclopropane-1-carboxylic acid (ACC) to alpha-ketobutyrate and ammonia (Honma and Shimomura, 1978, *Agric. Biol. Chem.* Vol.42 No.10: 1825-1813). The expression of the ACC deaminase enzyme in plants results in inhibition of the ethylene biosynthesis (Klee et al., 1991, *Plant Cell* Vol. 3, pp. 1187-1193) which affects ripening. pMON18131 was constructed by inserting the 1.1 kb ACC deaminase gene as an XbaI-BamHI fragment into pMON18103 (FIG. 16), replacing the glgC16 coding sequence. Thus, pMON19486 is comprised of, from 5' to 3',

the enhanced CaMV35S promoter, HSP70 intron, ACC deaminase coding sequences, and nopaline synthase polyadenylation region in a pUC backbone containing a  $\beta$ -lactamase gene for ampicillin selection in bacteria.

Stably transformed BMS calli were produced using particle gun bombardment to introduce pMON18131 as described in Example 3A. pMON18131 was bombarded in combination with EC9 (FIG. 19) into BMS cells. Resistant calli were selected on 20 ppb chlorsulfuron. The resistant calli were then assayed for expression of the ACC deaminase gene.

Chlorsulfuron resistant calli bombarded with pMON18131 were assayed by Western blot analysis. Seventeen of 24 lines examined showed high levels of ACC deaminase protein accumulation (~0.1% of total protein).

## Example 10

Production of Plants Using Vectors Containing the HSP70 Intron and Bialaphos Selection

pMON19477 (FIG. 24) contains the BAR gene from *S. hygroscopicus*. The BAR gene encodes a phosphinothricin acetyltransferase enzyme that can be used as a selectable marker by conferring resistance to bialaphos or phosphinothricin, the active ingredient in the herbicide BASTA (Fromm et al., 1990, *Bio/Technology* 8:833-839; De Block et al., 1987, *Embo. J.* 6:2513-2518; Thompson et al., 1987, *Embo. J.* 6:2519-2523). pMON19477 was constructed by inserting the BAR gene as a 0.6 kb BamHI-BclI fragment into the BamHI site in pMON19470 (FIG. 8). Thus, pMON19477 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, BAR coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

pMON19493 (FIG. 25) contains a "synthetic" *B.t.k.* gene consisting of 1.8 kb truncated gene encoding amino acids 1 to 615 of the *Bacillus thuringiensis* kurstaki CryIA(b) insect control protein described by Fischhoff et al. (1987) *Bio/Technology* 5: 807-813, translationally fused to the 1.8 kb 3' half of the CryIA(c) gene encoding amino acids 616-1177 (Adang et al. 1985, *Gene* 36: 289-300). Expression of the gene in plants results in insect resistance. pMON19493 was constructed by inserting the 3.6 kb "synthetic" *B.t.k.* gene coding sequence as a BglII fragment into the BamHI site in pMON19470 (FIG. 8). Thus, pMON19493 is comprised of, from 5' to 3', the enhanced CaM 35S promoter, HSP70 intron, "synthetic" *B.t.k.* coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

pMON19468 (FIG. 26) contains the *E. coli* GUS gene and can be used as a visible scoreable marker of transformation using histochemical staining. pMON19468 was constructed using the 1.8 kb BglII-EcoRI fragment containing the GUS gene from pMON8678 inserted into the BamHI-EcoRI site in the pMON19470 backbone. Thus, pMON19468 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, GUS coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

Embryogenic cultures were initiated from immature maize embryos of the "Hi-II" genotype (Armstrong et al., 1991, *Maize Genetic Newsletter* 65:92-93) cultured 18-33 days on N6 2-100-25-Ag medium (Chu et al., 1975, *Sci. Sin. Peking* 18:659-688) modified to contain 2 mg/L 2,4-dichlorophenoxyacetic acid, 180 mg/L casein hydrolysate, 25 mM L-proline, 10 uM silver nitrate, pH5.8, solidified

with 0.2% Phytigel™ (Sigma). These embryogenic cultures were used as target tissue for transformation by particle gun bombardment.

A 2:1:1 mixture of pMON19477, pMON19493, and pMON19468 plasmid DNAs was precipitated onto tungsten M10 particles by adding 12.5 ul of particles (25 mg/ml in 50% glycerol), 2.5 ul experimental plasmid DNA (1 ug/ul), 12.5 ul 1M calcium chloride, and 5 ul 0.1M spermidine, and vortexing briefly. The particles were allowed to settle for 20 minutes, after which 12.5 ul of supernatant was removed and discarded. Each sample of DNA-tungsten was sonicated briefly and 2.5 ul was bombarded into the embryogenic cultures using a PDS-1000 Biolistics particle gun (DuPont).

The tissue was transferred to fresh, nonselective medium the day after bombardment. Six days post-bombardment, the material was transferred to selective media containing 2 mg/L 2,4-dichlorophenoxyacetic acid, 10 uM silver nitrate, no casamino acids or proline, and 0.3 mg/L bialaphos. After 2–3 weeks, the cultures were transferred to fresh media which contained 1.0 mg/L bialaphos. The cultures were maintained on the 1.0 mg/L bialaphos media, transferred at 2–3 week intervals, until bialaphos-resistant calli could be distinguished. Seven bialaphos resistant calli were recovered from eight plates of embryogenic material.

Bialaphos resistant lines were bulked up and assayed for *B.t.k.* or GUS expression. All lines were tested for insecticidal activity in Tobacco Hornworm (THW) feeding assays to test for *B.t.k.* expression. Approximately 0.5 g of the embryogenic callus was fed to 10–12 THW larvae. Two lines, 284-5-31 and 284-6-41, were positive and showed significant lethality to the THW insects, indicating that the *B.t.k.* gene from pMON19493 had integrated into their genomes and was being expressed. All lines were also assayed for GUS expression using a histochemical assay (Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W., 1987, *Embo. J.* 6:3901–3907). Of the seven lines tested, only a single line, 284-8-31, showed any detectable blue staining indicative of GUS expression from pMON19468.

Plants were regenerated from all of the bialaphos resistant calli in a three step regeneration protocol. All regeneration was performed on 1 mg/L BASTA. Embryogenic tissue was incubated on each medium for about two weeks and then transferred to the medium for the next step (see Table 6 for regeneration media ingredients). The first two steps were carried out in the dark at 28° C., and the final step under a 16:8 hour photoperiod, ~70 uE m<sup>-2</sup> sec<sup>-1</sup> provided by cool-white fluorescent bulbs, at ~25° C. Small green shoots that formed on Regeneration Medium 3 in 100×25 mm Petri plates are transferred to Regeneration Medium 3 in 200×25 mm Pyrex™ or Phytatrays™ to permit further plantlet development and root formation. Upon formation of a sufficient root system, the plants were carefully removed from the medium, the root system washed under running water, and the plants placed into 2.5" pots containing Metro-mix 350 growing medium. The plants were maintained for several days in a high humidity environment, and then the humidity was gradually reduced to harden off the plants. The plants were transplanted from the 2.5" pots to 6" pots and finally to 10" pots during growth.

TABLE 6

Regen 1	Regen 2	Regen 3
MS salts (Sigma; 4.4 g/L 1.30 mg/L nicotinic	N6 salts (Sigma; 4.0 g/L 0.5 mg/L nicotinic	MS salts (Sigma; 4.4 g/L 1.30 mg/L nicotinic

TABLE 6-continued

Regen 1	Regen 2	Regen 3
acid 0.25 mg/L pyridoxine HC1 0.25 mg/L thiamine HC1 0.25 mg/L Ca-pantothenate 100 mg/L myo-inositol 1 mM asparagine 0.1 mg/L 2,4-D 0.1 μM ABA 20 g/L sucrose 2.0 g/L Phytigel™ pH 5.8	acid 0.5 mg/L pyridoxine HC1 1.0 mg/L thiamine HC1 2.0 mg/L glycine 60 g/L sucrose 2.0 g/L Phytigel™ pH 5.8	acid 0.25 mg/L pyridoxine HC1 0.25 mg/L thiamine H1 0.25 mg/L Ca-pantothenate 100 mg/L myo-inositol 1 mM asparagine 20 g/L sucrose 2.0 g/L Phytigel™ pH 5.8

All ingredients can be obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.

All corn plants regenerated from bialaphos resistant embryogenic calli were shown to express at least one of the genes that had been bombarded: *BAR*, *B.t.k.*, or *GUS*. Plants regenerated from the bialaphos resistant, THW negative callus lines were confirmed to be transgenic and expressing the *BAR* gene by BASTA leaf painting assays. Seedlings were assayed when 4–5 leaves had fully emerged from the whorl. A solution of 1% BASTA, 0.1% Tween20 was applied to the upper and lower surfaces of the first fully emerged leaf. The plants were scored three days after painting. The control plants showed yellowing and necrosis on the leaves, while the leaves from the resistant lines were green and healthy. This indicates not only that the *BAR* gene in pMON19477 was expressed in these plants, but also that the expression levels were high enough to confer resistance to the herbicide BASTA at the plant level.

Plants regenerated from the two lines that had shown THW activity, 284-5-31 and 284-6-41, were assayed for *B.t.k.* expression by whole plant feeding assays. Plants approximately 30" in height were inoculated with 100 European Corn Borer (ECB) eggs. Feeding damage was scored on a scale of 0 (no damage) to 9 (high level of leaf feeding damage) two weeks after inoculation. The control plants scored insect feeding ratings of 9. All plants from either line containing pMON19493 received ratings of zero; no ECB damage was present.

The ECB feeding studies indicate that the *B.t.k.* gene was expressed at high enough levels in the regenerated plants to impart insect resistance. To quantitate the level of expression, samples from the regenerated lines were assayed by ELISA. Eight plants regenerated from each callus line were analyzed. Plants from line 284-5-31 ranged in *B.t.k.* expression from 0.006 to 0.034% of total cellular protein, with an average value of 0.02%. Plants from line 284-6-41 ranged in *B.t.k.* expression from 0.005 to 0.05%, also with an average of 0.02% of total protein.

#### Example 11

Production of Plants Using Glyphosate Selection Vectors Containing the HSP70 Intron

pMON19640 (FIG. 12) contains a 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) gene. To form pMON19640 (FIG. 12), a 1.75 kb XbaI-EcoRI fragment containing the maize EPSPS coding sequence with two mutations (Gly144>Ala and Gly206>Asp) of mature peptide that confers tolerance to glyphosate herbicide was inserted into the corresponding restriction sites in pMON19470 (FIG. 8). Thus, pMON19640 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, EPSPS

coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

pMON19643 (FIG. 17) contains a gene fusion composed of the N-terminal 0.26 Kb chloroplast transit peptide sequence derived from the Arabidopsis thaliana SSU 1a gene (SSU CTP) (Timko et al., 1988, *The Impact of Chemistry on Biotechnology*, ACS Books, 279–295) and the C-terminal 1.3 Kb synthetic GOX gene sequence. The GOX gene encodes the enzyme glyphosate oxidoreductase which catalyzes the conversion of glyphosate to herbicidally inactive products, aminomethylphosphonate and glyoxylate. Plant expression of the gene fusion produces a pre-protein which is rapidly imported into chloroplasts where the CTP is cleaved and degraded releasing the mature GOX protein (della-Cioppa et al., 1986, *Proc. Natl. Acad. Sci. USA* 83: 6873–6877). pMON19643 (FIG. 18) was constructed by inserting the SSU-CTP—GOX fusion coding sequences into pMON19470 as a 1.6 kb BglII/EcoRI fragment into BamHI-EcoRI digested pMON19470 (FIG. 8). Thus, pMON19643 is comprised of, the from 5' to 3', enhanced CaMV35S promoter, HSP70 intron, SSU-CTP—GOX coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

Embryogenic cultures were initiated from immature maize embryos of the “Hi-II” genotype (Armstrong et al., 1991, *Maize Genetic Newsletter* 65:92–93) cultured 18–33 days on N6 1-100-25 medium (Chu et al., 1975, *Sci. Sin. Peking*, 18:659–688) modified to contain 1 mg/L 2,4-dichlorophenoxyacetic acid, 180 mg/L casein hydrolysate, 25 mM L-proline, and solidified with 0.2% Phytigel™ (Sigma). These embryogenic cultures were used as target tissue for transformation by particle gun bombardment.

A 1:1 mixture of PMON19640 and pMON19643 plasmid DNAs was precipitated onto tungsten M10 particles by adding 12.5 ul of particles (25 mg/ml in 50% glycerol), 2.5 ul experimental plasmid DNA (1 ug/ul), 12.5 ul 1M calcium chloride, and 5 ul 0.1M spermidine, and vortexing briefly. The particles were allowed to settle for 20 minutes, after which 12.5 ul of supernatant was removed and discarded. Each sample of DNA-tungsten was sonicated briefly and 2.5 ul was bombarded into the embryogenic cultures using a PDS-1000 Biolistics particle gun (DuPont).

One week after bombardment, cultures were transferred to fresh N6 1-0-25 medium (same as the initiation medium, except removing casein hydrolysate and adding 1 mM glyphosate). After two weeks growth on 1 mM glyphosate medium, cultures were transferred to the same base medium but with 3 mM glyphosate. Additional transfers were made at approximately 2 week intervals on 3 mM glyphosate medium. Glyphosate resistant calli were identified approximately 8–10 weeks post-bombardment, at a frequency of approximately 0.2–1.0 resistant calli per bombarded plate.

Plants were regenerated from glyphosate resistant calli as described for bialaphos resistant calli in Example 10, except that instead of 1 mg/L Basta either 0.01 mM glyphosate or no selective agents were added to the culture medium. Plants were analyzed for expression of pMON19643 by Western blot analysis. Leaf punches were taken from several individual plants regenerated from three independent glyphosate resistant calli. All three lines showed detectable levels of GOX gene expression. Four plants assayed from line 264-2-1 had a low but detectable level of GOX expression (approximately 0.002% of total protein). Five plants from line 269-1-1 showed higher GOX protein levels ranging from 0.04–0.06% of total protein. Lastly, 23 plants were assayed from line 292-5-1. GOX protein levels ranged from 0.05 to 0.1% of total protein. These plants sprayed with glyphosate at 29 oz./acre produced fully fertile plants. R<sub>1</sub> progeny of these plants were sprayed with glyphosate at 29,

58 and 115 oz/acre. One line of plants showed no vegetative damage at the highest application rate indicating glyphosate resistance at levels at which complete weed control would be achieved.

#### Example 12

##### Effect of the HSP70 Intron Alterations

##### A. Deletions within the HSP70 intron

Deletion 1 (FIG. 2) (SEQ ID NO:2) was created by digestion of pMON19433 with BsmI and NsiI, followed by treatment with T4 polymerase to create blunt ends, and religation. Deletion 2 (FIG. 3) (SEQ ID NO:3) was made similarly, except using digestion with BsmI and SnaBI. The effect on gene expression of the full length HSP70 intron versus the effect of Deletion 1 or Deletion 2 was compared in BMS particle gun transient assays as described in Example 2. As shown below, the introns with internal deletions increase GUS gene expression over the no intron control to a similar extent as the full length intron in pMON19433.

Intron	Relative GUS Expression
none	1X
HSP70 full length	32–51X
HSP70 deletion 1	14–38X
HSP70 deletion 2	14–30X

##### B. Alterations in 5' and 3' splice site consensus sequences

In the original polymerase chain reaction (PCR) synthesis of the HSP70 intron by polymerase chain reaction, a variant intron was also synthesized. This variant intron, when cloned adjacent to  $\beta$ -glucuronidase or luciferase, increases expression 4 fold relative to a no intron control but 10 fold less than the wild type HSP70 intron. The only significant difference in nucleotide sequence from that shown in SEQ ID NO:1 was a deletion of the adenine at nucleotide 19.

The HSP70 intron differs from the published (Brown, J. W. S., 1986, *Nuc. Acid Res.* 14:9949–9959) 5' splice site consensus sequence at two positions and from the 3' splice site consensus sequence at one position. The deletion of nucleotide 19 causes the variant HSP70 intron to diverge from the 5' splice site consensus sequence at four positions. Thus, the variant intron probably does not splice as efficiently as the wildtype intron and this may account for the difference in their effect on gene expression.

To address this question, variants of the HSP70 intron that contain perfect consensus sequences at the 5' splice junction, 3' splice junction, or both were constructed. The variants of the HSP70 intron were synthesized by PCR utilizing primers containing the desired changes to mutate the HSP70 intron splice sites to the 5' and/or 3' splice junction consensus sequences. Specifically, the 5' splice junction consensus primer contained nucleotides 1 to 26 of SEQ ID NO:1 except that nucleotide 15 and nucleotide 20 were each changed to adenine. The 3' splice junction consensus primer contained nucleotides that complement nucleotides 791 to 816, except that nucleotide 800 was changed to a guanine (cytosine in the primer).

The PCR products containing the variant HSP70 introns were digested with BglII and NcoI and cloned into pMON8677, analogously to the construction of pMON19433. Therefore, each vector contains, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron (original or variant),  $\beta$ -glucuronidase (GUS) coding sequence, and nopaline synthase polyadenylation region. They are all identical except for the intron. pMON19433 contains the original HSP70 intron, pMON19460 contains the 5' splice site consensus variant intron, pMON19463 contains the 3' splice site consensus variant intron, and pMON19464 contains a variant intron containing both 5' and 3' splice site consensus sequences.

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pMON19460, pMON19463, pMON19464, and pMON19433 were compared in transient gene expression assays in BMS cells as described in Example 2. As shown below, none of the variations in the HSP70 intron significantly altered GUS gene expression.

Splice junction			
Vector	5'	3'	Relative GUS expression
pMON19433	HSP70 wt	HSP70 wt	1X
pMON19460	consensus	HSP70 wt	1.1–1.4X
pMON19463	HSP70 wt	consensus	1.1–1.4X
pMON19464	consensus	consensus	1.6–1.7X

C. Increasing the number of exon sequences does not effect the HSP70 intron

The original HSP70 “intron” contains the entire intervening sequence as well as 10 bases of exon 1 and 11 bases of exon 2. Because the intron is placed in the 5' untranslated leader region between the enhanced CaMV35S promoter and coding sequence, those 21 bases of exon sequence are left behind in the leader. PCR primers that give 50 nucleotides of the 3' end of HSP70 exon 1 and/or 28 nucleotides of the 5' end of HSP70 exon 2 (Shah et al., 1985, In *Cell* and *Mol. Biol. of Plant Stress*. Alan R. Liss, Inc. pp.181–200) were used to synthesize introns containing different amounts of exon sequences to determine if extra HSP70 exon sequences would affect the splicing efficiency and ability to increase gene expression.

The PCR products containing the various HSP70 introns with different exon lengths were digested with BglII and NcoI and cloned into pMON8677, analogously to the construction of pMON19433. Therefore, each vector contains, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron plus surrounding exon sequences,  $\beta$ -glucuronidase (GUS) coding sequence, and nopaline synthase polyadenylation region. They are all identical except for the length of the HSP70 exon surrounding the intron.

These vectors were then compared in transient gene expression assays in BMS cells as described in Example 2. As shown below, none of the variations in the HSP70 intron significantly altered GUS gene expression.

Vector	Exon 1	Exon 2	Relative GUS Expression
19433	10 nt	11 nt	1X
19462	10 nt	28 nt	0.6–0.9X
19465	50 nt	11 nt	1.2–1.5X
19466	50 nt	28 nt	0.8–1.5X

## Example 13

## HSP70 Intron Increases Gene Expression in Wheat Cells

To test the effect of introns on gene expression in wheat cells, transient gene expression assays were performed. C983 wheat suspension cells (obtained from Dr. I. Vasil, Univ. of Florida) were plated and bombarded with  $\beta$ -glucuronidase vectors containing no intron (pMON8677), ADH1 intron (pMON8678), and the HSP70 intron (pMON19433) as described for corn suspension cells in Example 2. As shown below, the effect of the ADH1 and HSP70 introns on GUS expression in wheat cells is comparable to that in corn cells. The ADH1 intron vector produces higher levels of GUS expression than does the vector with no intron, but the HSP70 intron vector

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produces significantly higher levels of expression than the ADH1 intron vector.

Vector	Intron	Mean Relative GUS
pMON8677	none	1X
pMON8678	ADH1	2X
pMON19433	HSP70	6–9X

## Example 14

## The HSP70 Intron Increases Gene Expression in Rice

Rice tissue culture line 812M from rice strain 8706, an indica/japonica hybrid, was grown in MS medium. One day after subculture the cells were transferred to Whatman filters for particle gun bombardment. Bombardments were performed with  $\text{CaCl}_2$ /spermidine precipitated plasmid DNA using a PDS-1000 as described for BMS cells (Example 3). The cells were allowed to express the introduced genes for two days and then harvested.  $\beta$ -Glucuronidase (GUS) and luciferase (LUX) were assayed as described, supra. As shown in Table 7, in duplicate experiments the presence of the HSP70 intron in the 5' untranslated region increases average GUS expression relative to LUX expression about 10 fold over the expression observed with the vector without an intron.

TABLE 7

Effect of HSP70 Intron in Rice		
Vector	Intron	GUS/LUX
pMON8677	none	15.5
pMON19433	HSP70	150.7

## Example 15

## Expression of CP4 EPSPS Using HSP70 Intron Vectors

pMON19653 (FIG. 27) was constructed to test expression of the CP4 EPSPS gene (U.S. patent application Ser. No. 07/749,611 filed Aug. 28, 1991 incorporated herein by reference) in an HSP70 intron vector. A 1.7 kb BglII-EcoRI fragment containing the 300 bp chloroplast transit peptide from the Arabidopsis EPSPS gene (AEPSPS CTP) fused in frame to the 1.4 kb bacterial CP4 EPSPS protein coding region was cloned into BamHI-EcoRI digested pMON19470 to form pMON19653. Thus, pMON19653 is comprised of, from 5' to 3', the enhanced CaMV35S promoter, HSP70 intron, AEPSPS CTP/CP4 coding sequence, and nopaline synthase polyadenylation region in a pUC-like backbone containing an NPTII gene for kanamycin selection in bacteria.

pMON19653 was introduced into embryogenic cells in combination with pMON19643 and transformed calli selected on glyphosate medium as described in Example 11. Glyphosate resistant embryogenic callus were assayed by Western Blot analysis. The amount of CP4 protein expressed was determined by comparison to standards of *E. coli* produced protein. Nine lines were generated. The CP4 expression levels ranged from undetectable to 0.3% of the total protein in crude extracts made from the embryogenic callus, with an average value of 0.17%.

The above examples indicate that the use of vectors containing the HSP70 intron would be expected to enhance the expression in monocot plants of other DNA sequences encoding proteins.

## SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 3

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 816 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: double  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (synthetic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

AGATCTACCG TCTTCGGTAC GCGCTCACTC CGCCCTCTGC CTTTGTTACT GCCACGTTTC      6 0
TCTGAATGCT CTCTTGTTGT GTGATTGCTG AGAGTGTTT AGCTGGATCT AGAATTACAC      1 2 0
TCTGAAATCG TGTTCCTGCCT GTGCTGATTA CTTGCCGTCC TTTGTAGCAG CAAAATATAG      1 8 0
GGACATGGTA GTACGAAACG AAGATAGAAC CTACACAGCA ATACGAGAAA TGTGTAATTT      2 4 0
GGTGCTTAGC GGTATTTATT TAAGCACATG TTGGTGTTAT AGGGCACTTG GATTTCAGAAAG      3 0 0
TTTGCTGTTA ATTTAGGCAC AGGCTTCATA CTACATGGGT CAATAGTATA GGGATTTCATA      3 6 0
TTATAGGCGA TACTATAATA ATTTGTTTCGT CTGCAGAGCT TATTATTTGC CAAAATTAGA      4 2 0
TATTCCTATT CTGTTTTTGT TTGTGTGCTG TAAATTGTT AACGCCTGAA GGAATAAATA      4 8 0
TAAATGACGA AATTTTGATG TTTATCTCTG CTCCTTTATT GTGACCATAA GTCAAGATCA      5 4 0
GATGCACTTG TTTTAAATAT TGTGTCTGA AGAAATAAGT ACTGACAGTA TTTTGATGCA      6 0 0
TTGATCTGCT TGTGTGTTGT AACAAAAATTT AAAAAATAAAG AGTTTCCTTT TTGTTGCTCT      6 6 0
CCTTACCTCC TGATGGTATC TAGTATCTAC CAACTGACAC TATATTGCTT CTCTTTACAT      7 2 0
ACGTATCTTG CTCGATGCCT TCTCCCTAGT GTTGACCAGT GTTACTCACA TAGTCTTTGC      7 8 0
TCATTTTCATT GTAATGCAGA TACCAAGCGG CCATGG                                8 1 6

```

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 283 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (synthetic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

AGATCTACCG TCTTCGGTAC GCGCTCACTC CGCCCTCTGC CTTTGTTACT GCCACGTTTC      6 0
TCTGAATGTG ATCTGCTTGT TTGTTGTAAC AAAATTTAAA AATAAAGAGT TTCCTTTTTG      1 2 0
TTGCTCTCCT TACCTCCTGA TGGTATCTAG TATCTACCAA CTGACACTAT ATTGCTTCTC      1 8 0
TTTACATACG TATCTTGCTC GATGCCTTCT CCTAGTGTT GACCAGTGTT ACTCACATAG      2 4 0
TCTTTGCTCA TTTTCATTGTA ATGCAGATAC CAAGCGGCCA TGG                                2 8 3

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## ( 2 ) INFORMATION FOR SEQ ID NO:3:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 162 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

-continued

( i i ) MOLECULE TYPE: DNA (synthetic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGATCTACCG	TCTTCGGTAC	GCGCTCACTC	CGCCCTCTGC	CTTTGTTACT	GCCACGTTTC	60
TCTGAATGGT	ATCTTGCTCG	ATGCCTTCTC	CCTAGTGTTG	ACCAGTGTTA	CTCACATAGT	120
CTTTGCTCAT	TTCATTGTAA	TGCAGATACC	AAGCGGCCAT	GG		162

We claim:

1. An isolated DNA segment comprising in sequence:
  - (a) a promoter that functions in monocotyledonous plant cells;
  - (b) a non-translated leader DNA comprising an intron sequence including at least sufficient nucleotide sequence from the 5' end and from the 3' end of SEQ ID NO: 1 to splice said intron sequence; and
  - (c) a DNA sequence that in combination with (a) and (b) effects production of an RNA sequence; wherein the non-translated leader of (b) increases expression of the DNA sequence relative to wild-type expression.
2. The isolated DNA segment of claim 1, further comprising a 3' non-translated sequence that functions in plant cells to cause addition of a polyadenylated nucleotide sequence to the 3' end of said RNA sequence.
3. The isolated DNA segment of claim 1, wherein said intron sequence comprises at least about 162 nucleotides created by ligating a 5' end sequence and a 3' end sequence from SEQ ID NO:1.
4. The isolated DNA segment of claim 3, wherein said intron sequence is SEQ ID NO:3.
5. The isolated DNA segment of claim 3, wherein said intron sequence comprises at least about 283 nucleotides created by ligating a 5' end sequence and a 3' end sequence from SEQ ID NO: 1.
6. The isolated DNA segment of claim 5, wherein said intron sequence is SEQ ID NO:2.
7. The isolated DNA segment of claim 5, wherein said intron sequence is SEQ ID NO: 1.
8. The isolated DNA segment of claim 1, fused to said leader DNA further comprising between about 10 and about 50 additional nucleotides of an exon fused to said leader DNA.
9. The isolated DNA segment of claim 1, further comprising between about 11 and about 28 additional nucleotides of an exon fused to said leader DNA.
10. The isolated DNA segment of claim 1, further comprising between about 10 and about 50 additional nucleotides from the 3' end of HSP70 exon 1 and between about 11 and about 28 additional nucleotides from the 5' end of HSP70 exon 2 fused to said leader DNA.
11. The isolated DNA segment of claim 1, wherein said intron sequence comprises a splice site consensus sequence.
12. The isolated DNA segment of claim 11, wherein said intron sequence comprises a 5' splice site consensus sequence.
13. The isolated DNA segment of claim 11, wherein said intron sequence comprises a 3' splice site consensus sequence.
14. The isolated DNA segment of claim 11, wherein said intron sequence comprises a 5' splice site consensus sequence and a 3' splice site consensus sequence.
15. The isolated DNA segment of claim 11, wherein said intron sequence has an adenine nucleotide at position 15 of SEQ ID NO: 1.
16. The isolated DNA segment of claim 11, wherein said intron sequence has an adenine nucleotide at position 20 of SEQ ID NO: 1.
17. The isolated DNA segment of claim 11, wherein said intron sequence has a guanine nucleotide at position 800 of SEQ ID NO: 1.
18. The isolated DNA segment of claim 14, wherein said intron sequence has an adenine nucleotide at position 15, an adenine nucleotide at position 20, and a guanine nucleotide at position 800 of SEQ ID NO: 1.
19. The isolated DNA segment of claim 1, wherein said promoter comprises a plant DNA virus promoter.
20. The isolated DNA segment of claim 14, wherein said promoter is a CaMV35S promoter or an FMV promoter.
21. The isolated DNA segment of claim 1, wherein said DNA sequence encodes an EPSP synthase, a CP4 protein, an ACC-deaminase, a *B.t.* crystal toxin, a glgC16 protein, a plant viral coat protein or a GOX protein.
22. A method for the expression of a gene in a monocotyledonous plant, comprising introducing into the plant cell a DNA segment comprising in sequence:
  - (a) a promoter that functions in monocotyledonous plant cells;
  - (b) a non-translated leader comprising an intron sequence including at least sufficient nucleotide sequence from the 5' end and from the 3' end of SEQ ID NO: 1 to splice said intron; and
  - (c) a DNA sequence comprising said gene; wherein the DNA segment comprising (a), (b) and (c) produces an RNA sequence that effects expression of said gene.
23. The method of claim 22, wherein said DNA further comprises a 3' non-translated sequence that functions in monocotyledonous plant cells to cause addition of a polyadenylated nucleotide sequence to the 3' end of said RNA sequence.
24. The method of claim 22, wherein said intron sequence comprises at least about 162 nucleotides comprised by ligating a 5' end sequence and a 3' end sequence from SEQ ID NO: 1.
25. The method of claim 22, further comprising between about 10 and about 50 additional nucleotides of an exon fused to said leader DNA.
26. The method of claim 22, wherein said intron sequence comprises a splice site consensus sequence.
27. The method of claim 22, wherein said DNA sequence encodes an EPSP synthase, a CP4 protein, an ACC-deaminase, a *B.t.* crystal toxin, a glgC16 protein, a plant viral coat protein or a GOX protein.
28. The method of claim 22, wherein said plant is maize, wheat or rice.
29. A transgenic monocotyledonous plant comprising the DNA of claim 11.
30. The plant of claim 29, wherein said DNA further comprises a 3' non-translated sequence that functions in plant cells to cause addition of a polyadenylated nucleotide sequence to the 3' end of said RNA sequence.

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**31.** The plant of claim **29**, wherein said intron sequence comprises at least about 162 nucleotides obtained by ligating a 5° end sequence and a 3° end sequence from SEQ ID NO: 1.

**32.** The plant of claim **29**, further comprising between about 10 and about 50 additional nucleotides of an exon fused to said leader DNA.

**33.** The plant of claim **29**, wherein said intron sequence comprises a splice site consensus sequence.

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**34.** The plant of claim **29**, wherein said DNA sequence encodes an EPSP synthase, a CP4 protein, an ACC-deaminase, a *B.t.* crystal toxin, a glgC16 protein, a plant viral coat protein or a GOX protein.

**35.** The plant of claim **29**, wherein said plant is maize, wheat or rice.

\* \* \* \* \*

# Exhibit 5



US006350575B1

(12) **United States Patent**  
**Lusky et al.**

(10) **Patent No.:** **US 6,350,575 B1**  
(45) **Date of Patent:** **\*Feb. 26, 2002**

(54) **HELPER VIRUSES FOR THE PREPARATION OF RECOMBINANT VIRAL VECTORS**

(75) Inventors: **Monika Lusky**, Freiburg (DE); **Majid Mehtali**, Illkirch Graffenstaden (FR)

(73) Assignee: **Transgene S.A.**, Strasbourg (FR)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/563,239**

(22) Filed: **May 2, 2000**

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(63) Continuation of application No. 09/011,257, filed as application No. PCT/FR96/01200 on Jul. 30, 1996, now Pat. No. 6,066,478.

## (30) Foreign Application Priority Data

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(51) **Int. Cl.**<sup>7</sup> ..... **C12Q 1/68**; C12Q 1/70; C12N 15/861; C12N 15/64; C12N 5/10

(52) **U.S. Cl.** ..... **435/5**; 435/320.1; 435/69.1; 435/455; 435/456; 435/457; 435/462; 435/325; 435/366; 435/369; 435/6; 435/91.42

(58) **Field of Search** ..... 435/320.1, 69.1, 435/455, 456, 457, 325, 366, 369, 5, 6, 91.42, 462; 424/93.1, 93.2, 93.6

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*Primary Examiner*—David Guzo

(74) *Attorney, Agent, or Firm*—Burns, Doane, Swecker & Mathis, L.L.P.

## (57) ABSTRACT

Novel helper vectors are provided for complementing defective recombinant viral vectors, characterized in that they are provided with recombination sequences recognized by a recombinase. A complementation cell expressing the recombinase, and a method for preparing recombinant viral vectors as infectious viral particles for transferring and expressing genes of interest in a host organism or cell, are also provided. The invention is particularly suitable for use in gene therapy, especially in humans.

**34 Claims, 7 Drawing Sheets**



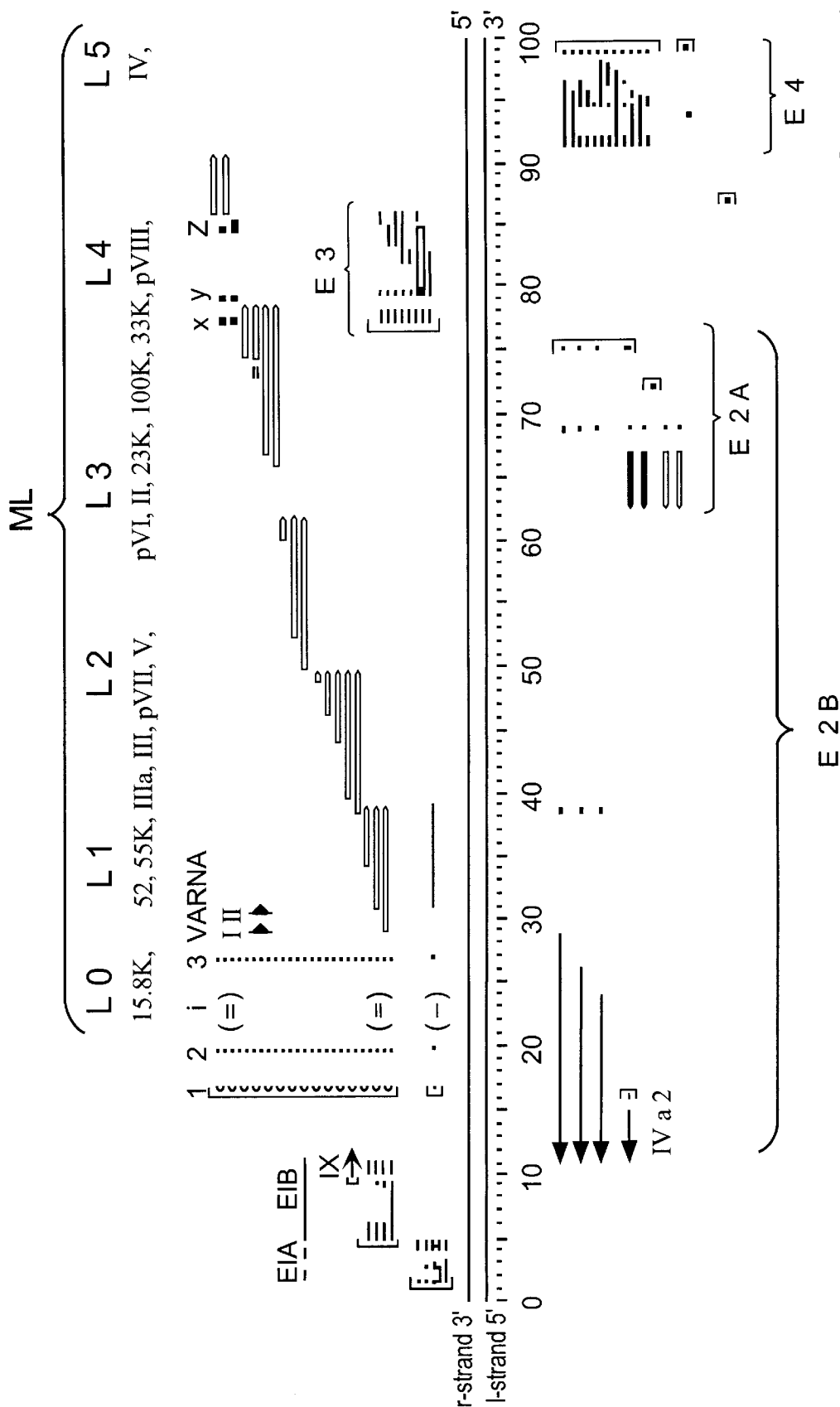
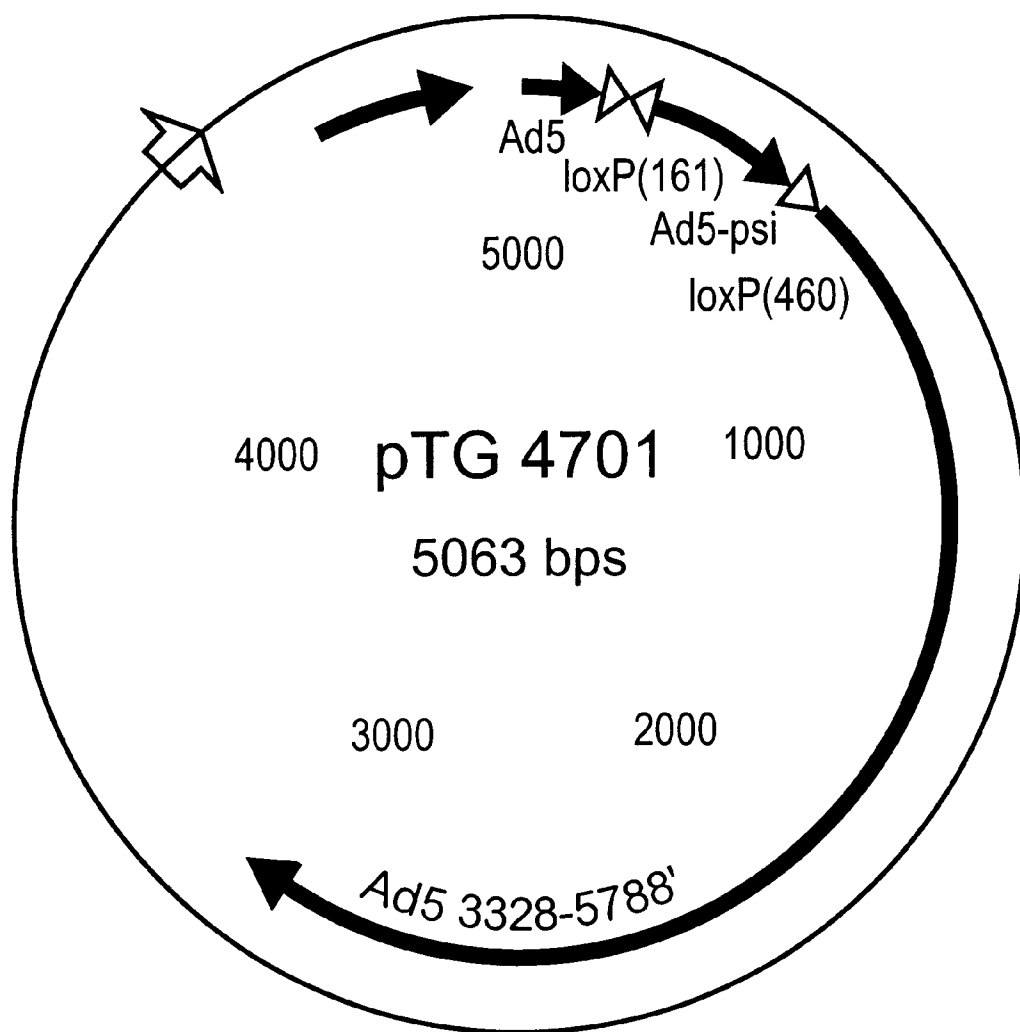


FIGURE 1

**FIG. 2**

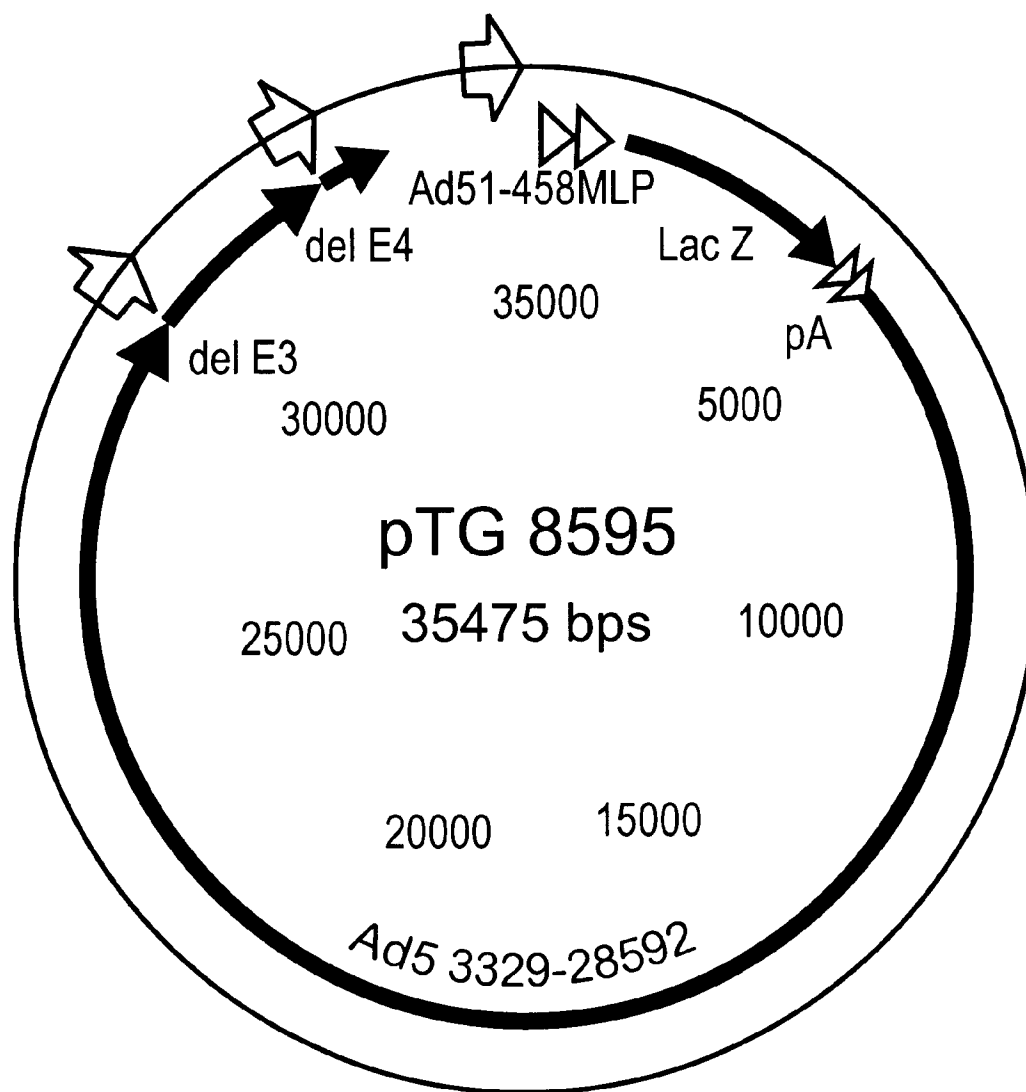


FIG. 3

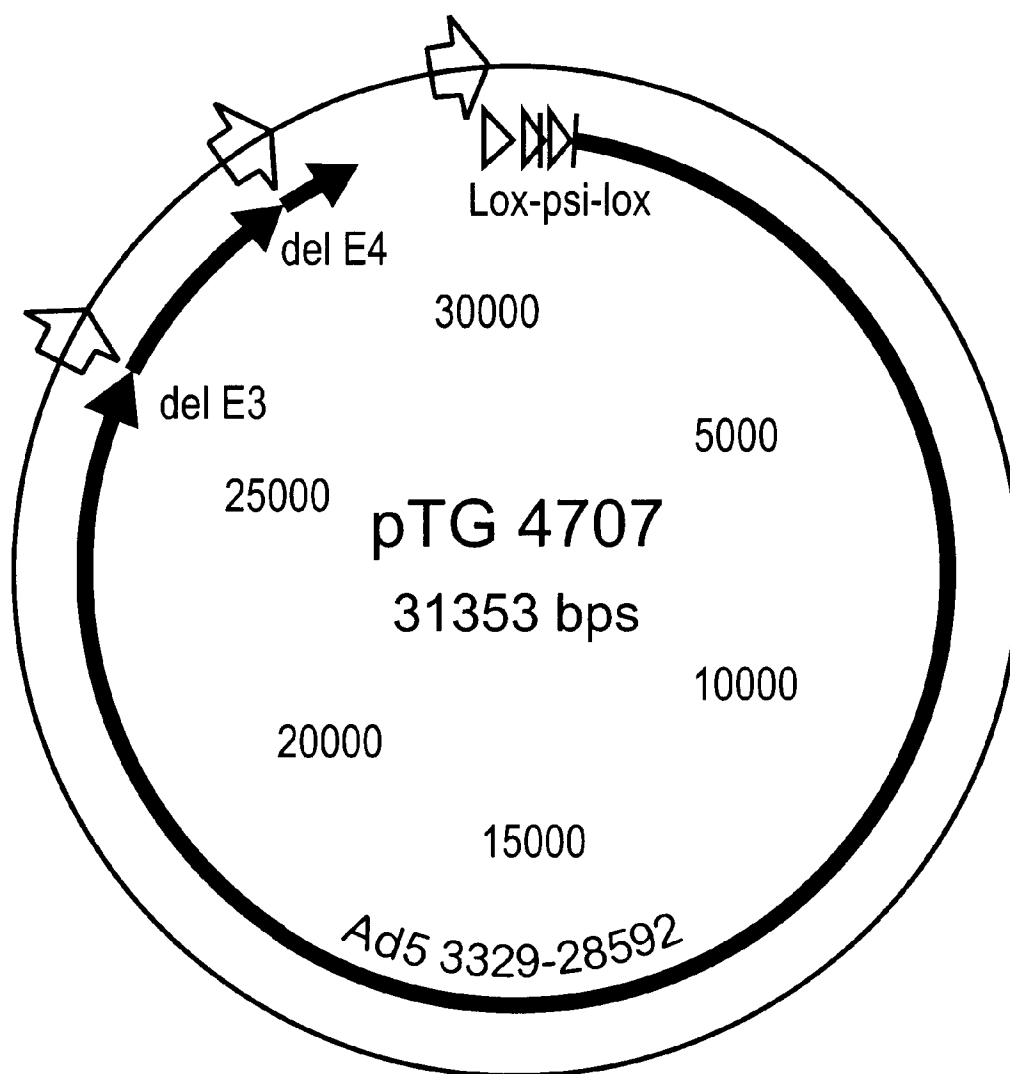


FIG. 4

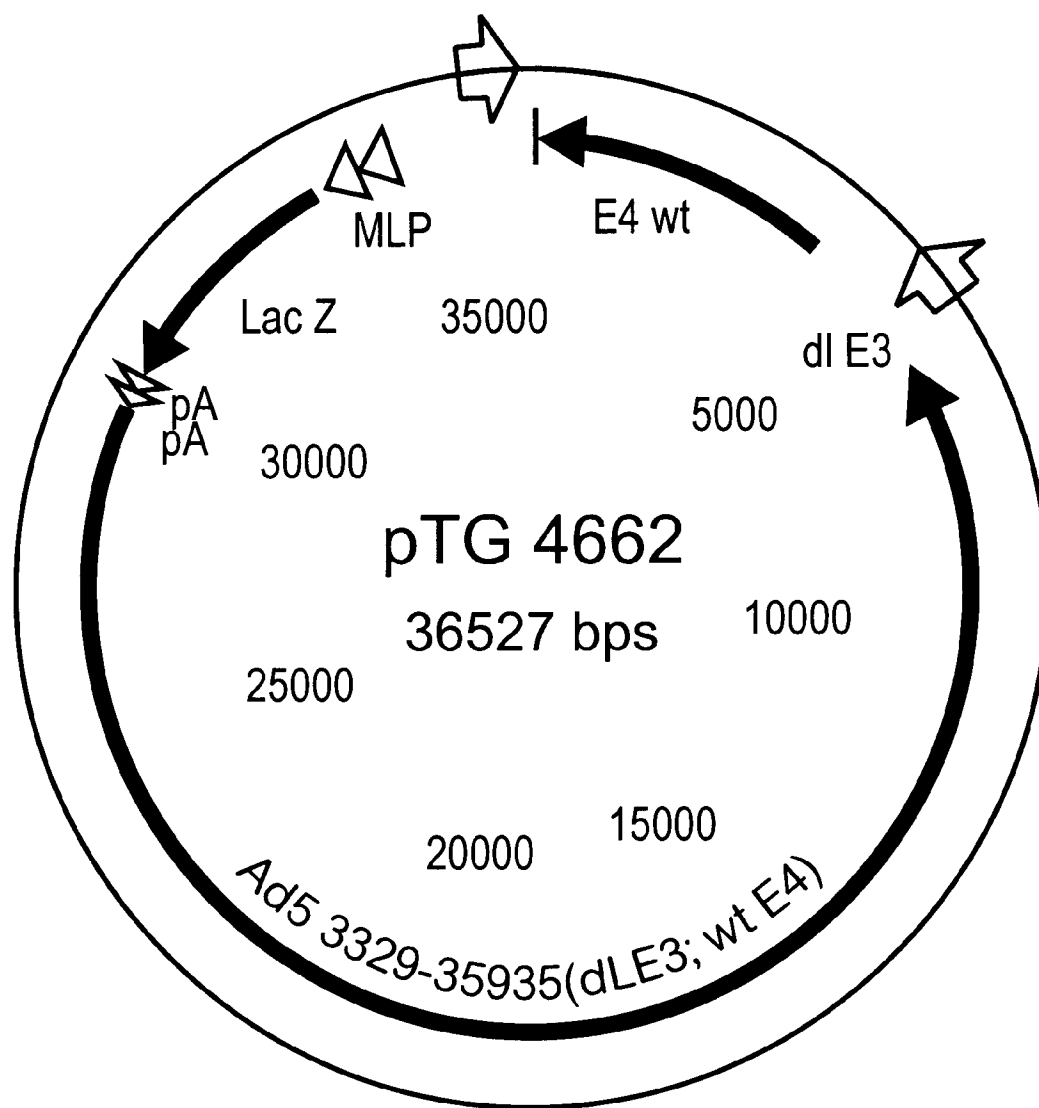


FIG. 5

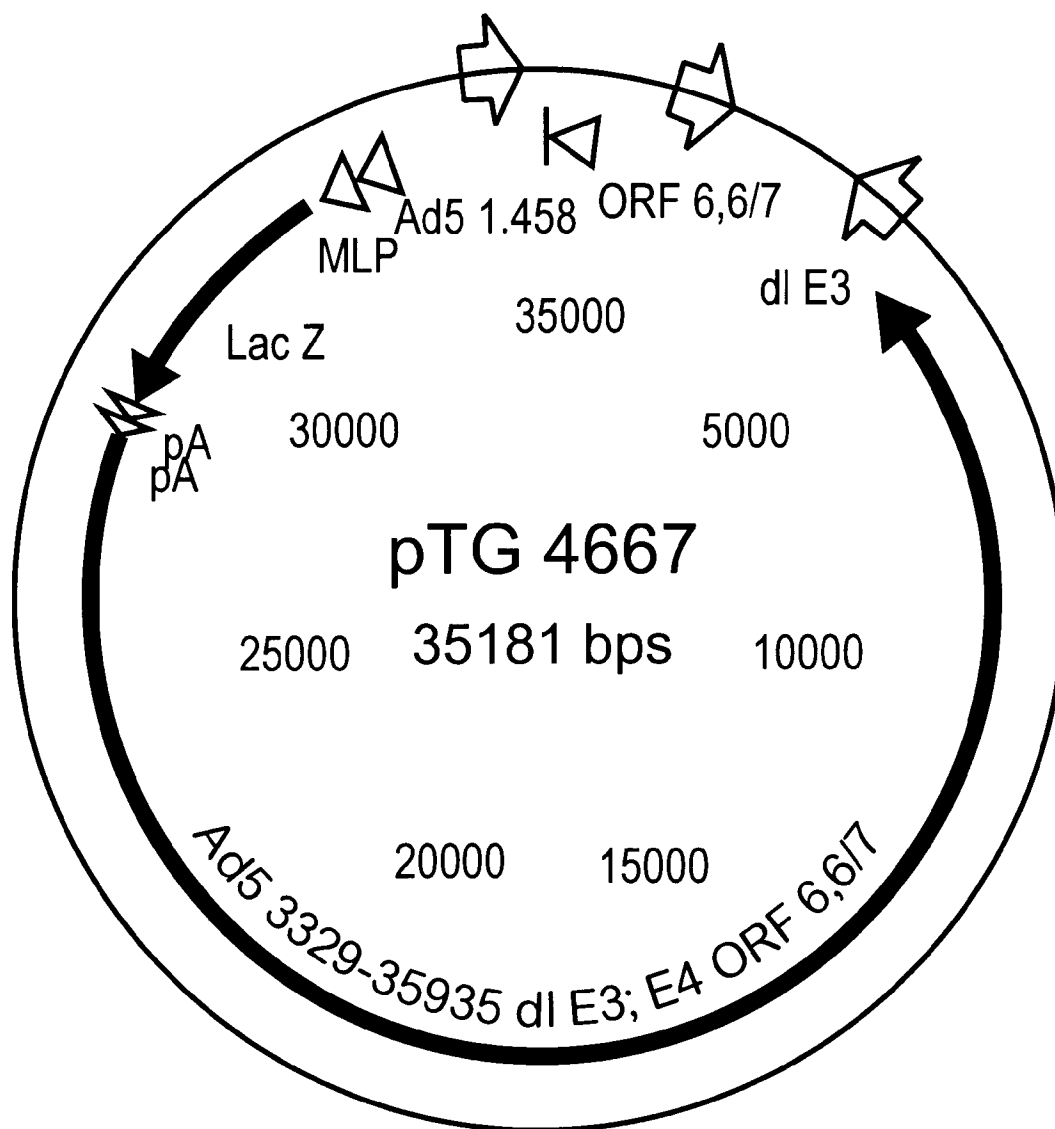


FIG. 6

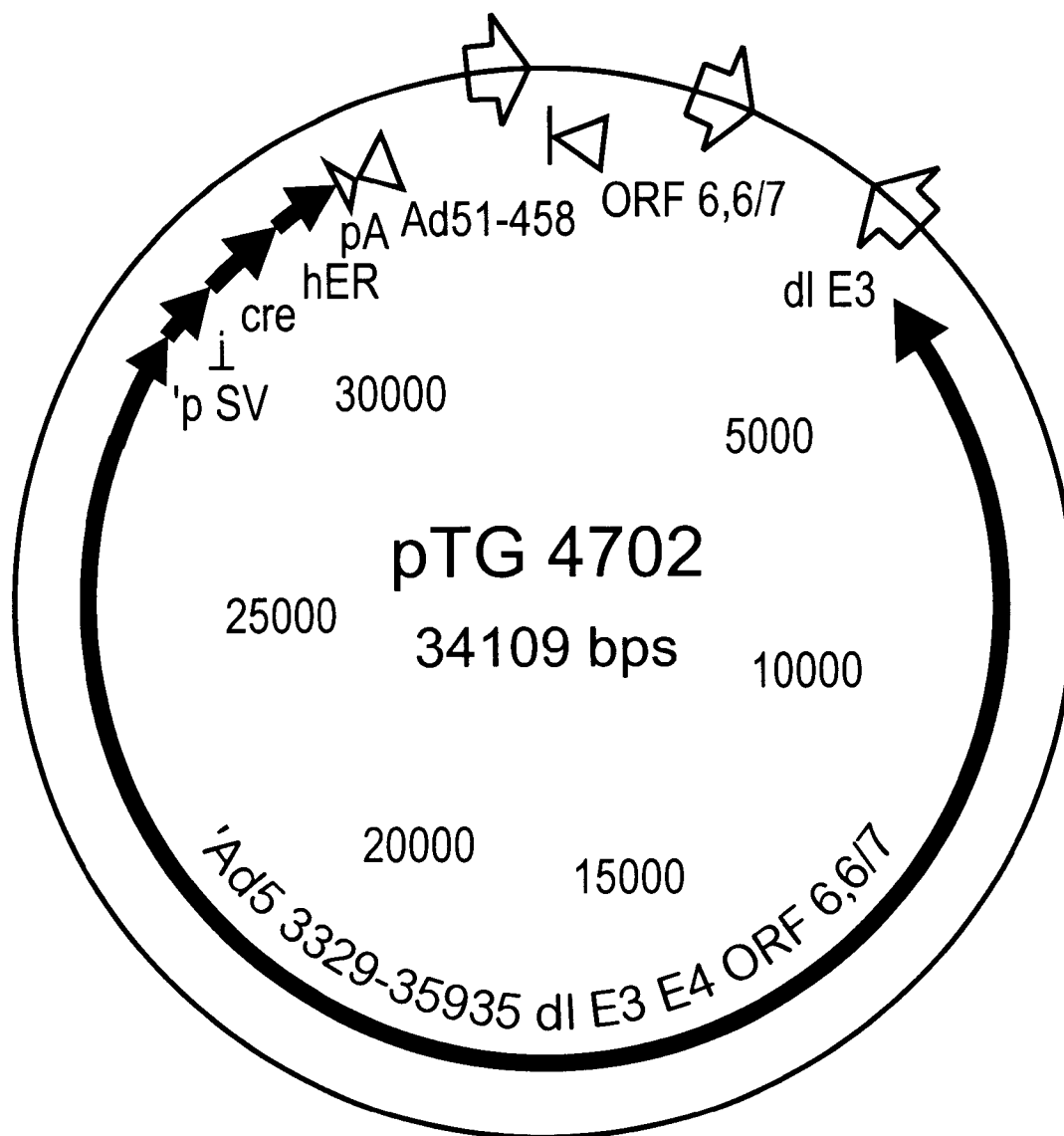


FIG. 7

## HELPER VIRUSES FOR THE PREPARATION OF RECOMBINANT VIRAL VECTORS

This application is a continuation of application Ser. No. 09/011,257, filed Mar. 9, 1998 now U.S. Pat. No. 6,066,478 which is a 371 application of PCT/FR96/01200, filed Jul. 30, 1996.

The present invention relates to novel helper vectors allowing defective recombinant viral vectors, which have the characteristic of being provided with recombination sequences recognized by a recombinase, to be complemented. It likewise relates to a complementation cell expressing the recombinase as well as a method of preparation of recombinant viral vectors in the form of infectious viral particles allowing the transfer and the expression of genes of interest in a cell or a host organism. The invention is of very particular interest for gene therapy prospects, especially in man.

The possibility of treating human diseases by gene therapy has passed in the course of a few years from the stage of theoretical considerations to that of clinical applications. The first protocol applied to man was initiated in the United States in September 1990 on a patient who was genetically immunodeficient because of a mutation affecting the gene coding for adenosine deaminase (ADA). The relative success of this first experiment encouraged the development of new gene therapy protocols for various genetic or acquired diseases (infectious diseases and especially viral diseases such as AIDS or cancers). The great majority of the protocols described until now employ viral vectors to transfer and express the therapeutic gene in the cells to be treated.

The interest in adenoviruses as gene therapy vectors has already been touched on in numerous documents of the prior art. In fact, the adenoviruses have a wide spectrum of hosts, are not very pathogenic and do not have the disadvantages connected with the retroviruses since they are nonintegrative and replicate equally in quiescent cells. By way of information, their genome is formed of a linear and double-stranded DNA molecule of approximately 36 kb carrying regions acting in cis (ITR 5' and 3' encapsidation region of the viral genome and ITR 3') and additionally about thirty genes, at the same time early genes necessary for viral replication and late structure genes (see FIG. 1).

The early genes are divided into 4 regions dispersed in the adenoviral genome (E1 to E4; E for early). They comprise 6 transcriptional units which have their own promoters. The late genes (L1 to L5; L for late) partly cover the early transcription units and are, for the majority, transcribed starting from the major late promoter MLP.

At the present time, all the adenoviral vectors used in gene therapy protocols are devoid of the major part of the E1 region essential for replication, in order to avoid their distribution in the environment and the host organism (first generation vectors). This deletion makes the viral genome deficient for replication. However, the E1 viruses can be propagated in a cell line which complements the E1 function to generate an infectious viral particle. The 293 line, established starting from human embryonic kidney cells, is currently used, in the genome of which is integrated the left 5' end of the type 5 adenovirus (Graham et al., 1977, J. Gen. Virol. 36, 59-72).

The majority of adenoviral vectors of the prior art comprise supplementary deletions. Certain of these have been introduced in the E3 region with the aim of increasing the cloning capacities but do not need to be complemented to the extent where the E3 region is nonessential for replication. More recently, second generation vectors have been

proposed in the literature. They conserve the in cis regions (ITRs and encapsidation sequences) and comprise important internal deletions aimed at suppressing the main part of the viral genes whose expression can be responsible for inflammatory responses in the host. In this respect, a minimal vector which is deficient for all of the coding viral regions represents a choice alternative.

The techniques of preparation of adenoviral vectors are widely described in the literature. Firstly, the complete genome is formed by homologous recombination in the 293 line (see especially Graham et Preveet, 1991, Methods in Molecular Biology, Vol. 7, Gene Transfer and Expression Protocols; Ed E. J. Murray, The Human Press Inc. Clinton, N.J.) or in *Escherichia coli* (technique described in the French Application No. 94 14470).

It is then necessary to propagate the vector in order to form a stock of viral particles containing it. This production step is critical and must allow high titers of infectious particles to be attained to be able to consider development on a large scale with a view to the preparation of clinical batches. If the first generation adenoviral vectors can be propagated relatively easily in the 293 cell line, the only complementation line described to date and capable of efficiently expressing E1, such is not the case for second generation vectors. In fact, according to the same basic principle, such a vector must be complemented for the essential functions which it cannot express.

The complementation can be provided "in trans" by the cell line employed (designated complementation cell line). It is then necessary to have new lines complementing several essential viral functions (E1 and E2, E1 and E4 or E1, E2 and E4). However, the various attempts carried out until now give the impression that the coexpression of several adenoviral regions is potentially toxic, such that the line risks not being optimal in terms of growth capacity and yield of viral particles, these two criteria being indispensable for industrial exploitation.

Another alternative is based on the use of a supplementary viral element, called "helper virus" introduced into the line at the same time as the adenoviral vector (two-component system). At the present time, an adenovirus from which the E1 region has been deleted and which is capable of synthesizing the expression products of other adenoviral regions is currently used. The co-transfection of such a helper virus and of an adenoviral vector in the 293 line allows the formation of viral particles.

However, a major disadvantage of this method is that the cells produce a mixed population of viral particles, one type comprising the recombinant vector and the other type the helper virus. In practice, the preparations mainly contain viral particles of helper virus, the contamination being able to reach and even exceed 90%. The presence of the helper virus is not desirable in the context of a therapy applied to man and therefore necessitates the employment of cumbersome and costly physical separation techniques, such as ultracentrifugation. In addition, this technology is not very well adapted to the production of vectors of complex structure, such as the second generation vectors, to the extent where, very often, the helper virus has a selective advantage (more rapid replication).

The unresolved problem to this day of the production of recombinant adenoviral vector particles with a high titer is an obstacle to the development of gene therapy.

A novel helper virus has now been constructed by insertion of direct repetitions on both sides of the encapsidation region. The action of a recombinase recognizing them involves the excision of the genetic material situated



between them. This deletion does not have any notable consequence on the expression of the viral genes but limits the encapsidation of the helper virus in a viral particle. Thus, the employment of the two-component procedure described above in a cell line expressing the recombinase will allow preparations enriched in adenoviral vector particles of interest to be produced.

The present invention follows from the perfection of a genetic technique based on the use of recombination sequences and of a recombinase to produce mainly the recombinant viral vector and to limit the contamination by the helper virus. The aim of the present invention is to put at the disposal of the public a novel helper virus able to express the genes which it carries (that is to say capable of exercising its function of trans-complementation) but unable to be propagated in the presence of a recombinase. The solution provided by the present invention combines safety of use (preparation enriched in recombinant viral vector), simplicity (production in a conventional cell line in the presence of recombinase) and efficiency (high titer compatible with industrial needs). It is very particularly adapted to the production of second generation adenoviral vectors.

For this reason the present invention relates to a helper virus for the production of a recombinant viral vector defective for replication, characterized in that it comprises a first recombination sequence at 5' and a second recombination sequence at 3' of a region essential for the propagation of said helper virus; said recombination sequences being recognized by a recombinase.

The term "helper virus" designates a vector able to trans-complement in full or in part a recombinant viral vector defective for replication. It is thus able to produce at least one polypeptide, early and/or late, which the recombinant vector cannot produce itself and which is necessary for the formation of a viral particle. "In full" signifies that the helper virus is capable of complementing the whole of the viral genome essential for replication of which the recombinant viral vector is devoid and "in part" signifies that the complementation is limited to a part of the defective functions.

In the context of the present invention, a helper virus derives from a natural virus such as found in nature as well as from a virus whose genome comprises modifications with respect to that of the parent virus from which it is descended. These modifications can have been introduced in vitro by genetic engineering techniques. They can be different (deletion, mutation and/or addition of one or more nucleotides) and localized in the encoding regions of the viral genome or outside of these. The modification can, for example, allow one or more gene(s) essential for viral replication to be inactivated with the aim of likewise rendering it defective that is to say incapable of autonomous replication.

The human adenoviruses of serotype C and, more particularly, of type 2, 5 or 7 represent particularly preferred viruses in the context of the invention. However, it is likewise possible to resort to other adenoviruses, especially of animal origin (canine, bovine, murine, avian, ovine, porcine or simian). It is more particularly possible to mention the canine adenoviruses CAV-1 or CAV-2, avian viruses DAV or even bovine viruses Bad of type 3 (Zakharchuk et al., 1993, Arch. Virol., 128, 171-176; Spibey and Cavanagh, 1989, J. Gen. Virol., 70, 165-172; Jovenne et al., 1987, Gene, 60, 21-28; Mittal et al., 1995, J. Gen. Virol., 76, 93-102). However, it may also be of interest to have a helper virus derived from a poxvirus (vaccinia virus, fowlpox, canarypox . . . ), retrovirus, herpesvirus, cytomegalovirus,

adenovirus-associated virus (AAV) or even at a hybrid virus comprising fragments of different origin.

The characteristic of the helper virus according to the invention is that it comprises at least two recombination sequences inserted at 5' and at 3' of a sequence essential for its propagation. An essential sequence designates all or part of a viral gene, elements necessary for the expression such as a promoter or, in a preferred manner, elements acting in cis (ITR, LTR, encapsidation sequence . . . ). By way of information, the first and/or second recombination sequences can be positioned in the interior or immediately at 5' and 3' of the essential region up to about several hundreds of bp.

In the sense of the present invention, a "recombination sequence" is formed by a nucleic acid sequence (DNA or RNA) recognized by a recombinase able to induce a recombination event. Usually, a recombination sequence has at least 10 base pairs (bp), advantageously 15 to 80 bp, preferably 20 to 60 bp and, in a very preferred manner, 30 to 50 bp. According to an advantageous embodiment, a helper virus according to the invention comprises two copies of an identical or closely similar recombination sequence (at least 70% sequence identity and, in a preferred manner at least 90%). For this reason we shall talk of sequence repetitions. In this respect, the repetition can be reversed (the two recombination sequences present in the helper virus have a reverse orientation with respect to one another, one being in the direction 5' to 3' and the other 3' to 5') or direct (same orientation 5' to 3' or 3' to 5'). This second form will be preferred.

The recombination is accompanied by a pairing of the recombination sequences, by a cleavage of a target sequence at their level and by a ligation of the cleaved ends. The enzyme able to promote the recombination is designated "recombinase". The recombination between direct repetitions leads to the excision of sequences between them. On the other hand, recombination between reversed repetitions involves the reversal of the genetic material located between them.

Generally speaking, the recombination sequences and the recombinases are described in the literature accessible to the person skilled in the art. They can be of any viral, phagic, prokaryotic or eukaryotic origin (yeast, fungus or even higher eukaryote). In addition, they can be obtained by the conventional molecular biology techniques (cloning, amplification by chain reaction (PCR for Polymerase Chain Reaction) or by chemical synthesis.

As preferred examples, mention will be made of loxP recombination sequences (described in the identifier of sequence SEQ ID NO.: 1), FRT (SEQ ID NO.: 2) and R (SEQ ID NO.: 3) recognized by the recombinases CRE, FLP and R, respectively (see, for example, the review article Kilby et al., 1993, TIG 9, 413-420).

A helper virus particularly adapted to the present invention is derived from the genome of an adenovirus and comprises the ITRs 5' and 3', an encapsidation region and at least one viral gene selected from the genes of the E1, E2, E4 and L1-L5 regions and defective in the recombinant adenoviral vector as well as a first recombination sequence at 5' and a second recombination sequence at 3' of the encapsidation region. Preferably, they are formed by loxP sequences positioned in the same orientation with respect to one another. According to a first variant of interest, the encapsidation region can be attenuated (reduced capacity for encapsidation) to favor the encapsidation of the recombinant viral vector. The attenuation can be obtained by deletion of a part of the encapsidation region. The means of attenuating an encapsidation region are indicated in the Application WO 94/28152.

According to a second variant of interest, the helper virus can include an expression cassette of a recombinase, and allowing especially an inducible expression or the production of an inactive recombinase activatable according to needs (defined below). The insertion takes place in an appropriate region of the helper virus and, preferably, outside of the localized region between the recombination sequences.

According to a particular embodiment intended to increase the safety aspect, a helper virus according to the invention can comprise a first recombination sequence and a second recombination sequence at 5' and at 3' of several regions essential to its propagation. For reasons of simplicity of employment, the case will be preferred where the recombination sequences are identical or related so as to be recognized by an identical recombinase.

The present invention likewise relates to a complementation cell line comprising a DNA fragment coding for a recombinase. It can be generated from various cell lines by introduction of appropriate portions of the viral genome and of the fragment in question. A line able to complement the E1 and/or E4 function of an adenovirus is more particularly preferred. Mention will be made of the lines 293 (Graham, 1997 *supra*) and 1653 (described in the Application WO 94/28152) modified by the introduction of the DNA fragment coding for a recombinase.

All the standard means for introducing a nucleic acid into a cell can be employed in the context of the present invention (synthetic, viral, plasmid vector, naked DNA . . . ). Of course, said DNA fragment can be integrated into the cell genome or remain in the episome state. For the aims of the present invention, it can comprise the elements necessary for its expression. These will be, preferably, elements conferring an inducible expression in response to an inducer. Such elements are known to the person skilled in the art. It is possible to mention, by way of information, promoters inducible by metals (promoter of the metallothioneine gene), by hormones (promoter comprising elements responding to glucocorticoids GRE, to progesterones PRE, to estrogens ERE . . . ), by viral inducers (promoter comprising the TRA or RRE sequence responding respectively to the TAT or REV protein of the human immunodeficiency virus HIV) or by various cellular inducers (promoter comprising the UAS-Gal4 sequence (for Upstream Activating Sequence Gal4) responding to Gal4 or the operators of the tetracycline bacterial operon responding positively to the tetracycline activator tTA).

In addition, a DNA fragment in use in the present invention codes for a recombinase able to recognize the recombination sequences present in a helper virus according to the invention. It is preferred to employ a recombinase selected from the group formed by CRE, FLP and R. However, it is equally possible to resort to a DNA fragment coding for a homolog of a recombinase whose sequence is modified with respect to the native sequence but exercising a similar or improved function. These modifications can result from the deletion, addition or substitution of one or more nucleotide(s). They can likewise be a hybrid protein resulting from the fusion of polypeptides from various origins, especially a polypeptide having a recombinase activity and the other a linking region. A recombinase particularly adapted to the present invention is formed by a hybrid protein, designated CRE-ER, resulting from the fusion of the recombinase CRE and of the linking region to the ligand of the human estrogen receptor; (Metzger et al., 1995, *Proc. Natl. Acad. Sci. USA* 92). The latter insofar as such is inactive and its biological activity is activated in the presence of a hormonal ligand such as estradiol.

The invention likewise relates to a process for preparing a viral particle comprising a recombinant viral vector, which comprises the following steps:

- (a) Preparing a recombinant viral vector deficient for replication;
- (b) Preparing a helper virus according to the invention;
- (c) Introducing the recombinant viral vector and the helper virus into an appropriate cell line;
- (d) Culturing said cell line under appropriate conditions to allow the production of the viral particle in the presence of a functional recombinase able to recognize said first and second recombination sequences; and
- (e) Recovering the viral particle in the cell culture.

In the sense of the present invention, a defective recombinant viral vector derives from a virus in the genome of which certain sequences have been deleted, rendered nonfunctional, mutated or even substituted by other sequences and, more particularly, a heterologous DNA fragment (normally not present in the parent virus). The insertion takes place in an appropriate region of the viral genome, so as to allow its expression in a host cell. A host cell is formed by any eukaryotic cell infectable by a viral particle containing said recombinant viral vector.

The heterologous DNA fragment in use in the present invention can be descended from a eukaryotic organism, from a prokaryote or from a virus other than that in which it is inserted. It can be isolated by any technique conventional in the field of the art, for example by cloning, PCR or chemical synthesis. It can be a fragment of genome type (comprising all or part of the whole of the introns), of complementary DNA type (cDNA, devoid of intron) or of mixed type (comprising all or part of at least one intron). In addition, it can code for an antisense RNA and/or a messenger RNA (mRNA) which will then be translated into a polypeptide of interest, the latter being able to be (i) intracellular, (ii) membranous present at the surface of the host cell or (iii) secreted into the external medium. In addition, it can be a polypeptide as found in nature (native) or a portion of the latter (truncated) or equally a chimeric polypeptide arising from the fusion of sequences of various origins or even mutated and having improved or modified biological properties.

In the context of the present invention, it can be advantageous to use a DNA fragment coding for a cytokine (interleukin including IL-2, interferon, colony-stimulating factor . . . ), a cell or nuclear receptor, a ligand, a clotting factor (factor VII, factor VIII, factor IX . . . ), CFTR protein (Cystic Fibrosis Trans-membrane Conductance Regulator), insulin, dystrophin, a growth hormone, an enzyme (renin urease, thrombin . . . ), an enzyme inhibitor (inhibitor of a viral protease,  $\alpha$ 1-antitrypsin . . . ), a polypeptide with antitumor effect (product of suppressor genes of tumors, polypeptide stimulating the immune system . . . ), a polypeptide able to inhibit or slow down the development of a bacterial, viral or parasitic infection (antigenic polypeptide, trans-dominant variant . . . ) an antibody, a toxin, an immunotoxin and finally a label (luciferase,  $\beta$ -galactosidase, product conferring resistance to an antibiotic . . . ). Of course, this list is not limiting and other genes can likewise be employed.

Advantageously, the heterologous DNA fragment is placed under the control of elements necessary for its expression in the host cell. "Necessary elements" designates all of the elements allowing the transcription of said DNA fragment to RNA (antisense RNA or mRNA) and the translation of the mRNA to polypeptide. These elements

comprise a regulatable or constitutive promoter, which can be heterologous or on the contrary homologous to the parent virus. It is possible to mention, as examples, the promoter of the human or murine PGK gene (Phospho Glycerate Kinase), the early promoter of the SV40 virus (Simian Virus), the LTR of RSV (Rous Sarcoma Virus), the TK promoter (Thymidine Kinase) of the HSV-1 virus (Herpes Simplex Virus) and the adenoviral promoters E1A and MLP. The necessary elements can, in addition, include additional elements (intron sequence, secretion signal sequence, nuclear localization sequence, translation initiation site, transcription termination poly A signal . . . ). Although it is not a preferred variant, it is indicated that the viral vector can likewise comprise a DNA fragment coding for the recombinase.

It is within reach of the person skilled in the art to generate a recombinant viral vector in use in the present invention. He will quite certainly know how to adapt the technology as a function of the specific data (type of vector, heterologous DNA fragment . . . ). According to a preferred variant, the vectors capable of being employed in the context of the present invention are recombinant adenoviral vectors defective for all of the viral functions or even all of the functions with the exception of E4. Such vectors are described in the International Application WO 94/28152.

A helper virus according to the invention is obtained by insertion in a viral genome of a first and a second recombination sequence on both sides of a region essential for replication and, preferably, of the encapsidation region, it being possible for the latter to be attenuated or nonattenuated. The person skilled in the art knows the regions essential for the replication of a virus and is able to carry out such a construction by applying the classical techniques of molecular biology. According to the variant mentioned above, it can likewise comprise a DNA fragment coding for a recombinase and, especially, the CRE-ER hybrid. According to a preferential embodiment, a helper virus according to the invention and the recombinant viral vector which it allows production of derive from the same parent virus and, in a very preferred manner, from an adenovirus.

After the actual construction step, the helper virus and the recombinant viral vector are introduced into an appropriate cell line. All the standard means for introducing a nucleic acid into a cell can be used in the context of the present invention, for example transfection, electroporation, microinjection, lipofection, adsorption and fusion of protoplasts. It is indicated that they can be co-introduced (concomitant fashion) or introduced separately (the helper virus according to the invention previously or subsequently to the recombinant viral vector).

Although any cell line can be employed in the context of the present invention, a complementation line is especially preferred. Recourse will be had to a line of the prior art (293, 1653 . . . ) when a recombinant viral vector or a helper virus comprising the DNA fragment coding for the recombinase is employed. On the other hand, when this is not the case, use will be made of a complementation cell line according to the invention.

After transfection, the cell line is cultured under appropriate conditions to allow the production of viral particles. A procedure according to the invention can, in addition, comprise an amplification step previous to the culture step in the presence of the functional recombinase. The aim of this step is to increase the quantities of helper virus and of recombinant viral vector in order to improve the yields. It can be carried out by culture in any permissive line or in the appropriate line in use in the present invention before the addition, expression or activation of the recombinase.

This first culture step is followed by a second step carried out in the presence of a functional recombinase able to recognize said first and second recombination sequences. In the context of the procedure according to the invention, this recombinase can be added to the cell culture, for example in substantially pure form. However, according to another very preferred and already mentioned variant, the recombinase is produced by one of the constituents of the procedure according to the invention, namely the recombinant viral vector or, in a preferred manner, the helper virus or the cell line. Once produced in functional form, the recombinase will cause the excision of the essential region of the helper virus localized between the recombination sequences, with the aim of preventing or reducing its propagation.

In addition, when a recombinase is employed whose expression is inducible by an inducer or the CRE-ER hybrid protein whose biological activity is dependent on a hormonal ligand, the culture step in the presence of the functional recombinase is carried out by the addition to the culture medium of the inducer or the ligand.

According to an advantageous embodiment intended to increase the safety of a procedure according to the invention, the helper virus and the recombinant viral vector are defective and can be conversely complemented, in total or in part. A variant of interest consists in employing (i) a helper adenovirus according to the invention defective for the functions E1 and E4 and comprising the loxP sequences at 5' and at 3' of the encapsidation region, (attenuated or nonattenuated) (ii) a recombinant vector defective for all the functions with the exception of E4 and (iii) a 293 cell line producing the CRE-ER hybrid recombinase. According to another advantageous alternative, a procedure according to the invention employs (i) a helper adenovirus according to the invention defective for the functions E1 and E4, comprising the loxP sequences at 5' and at 3' of the encapsidation region (attenuated or nonattenuated) and producing the CRE-ER hybrid recombinase, (ii) a recombinant vector defective for all of the functions with the exception of E4 and (iii) a conventional cell line 293.

The viral particles are recovered from the cell culture, from the medium or after lysis of the cells. Advantageously, a procedure according to the invention comprises an additional step of purification of the recombinant viral vector particles. Although the choice of the technique is wide and within the reach of the person skilled in the art, it is possible to mention more particularly ultracentrifugation on a cesium chloride or sucrose gradient.

Finally, the invention likewise relates to a procedure for preparing a viral particle comprising a recombinant viral vector by means of a helper virus, according to which the ratio viral particles of recombinant adenoviral vector to those of helper virus is greater than 50%, advantageously greater than 60%, preferably greater than 70% and, in a very preferred manner, greater than 80%.

The invention likewise relates to a recombinant viral vector particle obtained by a procedure according to the invention as well as to a eukaryotic host cell according to the invention. Said host cell is advantageously a mammalian cell and, preferably, a human cell and can comprise said vector in integrated form in the genome or in nonintegrated form (episome). It can be a primary or tumor cell of hematopoietic (totipotent stem cell, leukocyte, lymphocyte, monocyte or macrophage . . . ), muscular, pulmonary, tracheal, hepatic, epithelial or fibroblast origin.

The invention likewise relates to a pharmaceutical composition comprising as therapeutic or prophylactic agent a recombinant viral vector particle obtained by a procedure

according to the invention or a eukaryotic host cell according to the invention, in combination with a carrier acceptable from a pharmaceutical point of view. The composition according to the invention is intended in particular for the preventive or curative treatment of diseases such as:

- genetic diseases (hemophilia, mucoviscidosis, diabetes or myopathy, that of Duchène and Becker . . . ),
- cancers, such as those induced by oncogenes or viruses, viral diseases, such as hepatitis B or C or AIDS (acquired immunodeficiency syndrome resulting from infection by HIV), and
- recurrent viral diseases, such as viral infections caused by the herpesvirus.

A pharmaceutical composition according to the invention can be produced in a conventional manner. In particular, a therapeutically efficacious quantity of a therapeutic or prophylactic agent is combined with a carrier such as a diluent. A composition according to the invention can be administered by aerosol, locally or even systemically. The routes of administration envisaged within the context of the present invention can be intragastric, subcutaneous, intracardiac, intramuscular, intravenous, intraperitoneal, intratumor, intrapulmonary, nasal or intratracheal. The administration can take place in a single or repeated dose one or more times after a certain delay interval. The appropriate route of administration and the appropriate dose vary as a function of various parameters, for example of the individual or of the disease to be treated or even of the recombinant gene(s) to be transferred. In particular, the viral particles according to the invention can be formulated in the form of doses of between  $10^4$  and  $10^{14}$  pfu (plaque-forming units), advantageously  $10^5$  and  $10^{13}$  pfu and, preferably,  $10^6$  and  $10^{11}$  pfu. The formulation can likewise include an adjuvant which is acceptable from a pharmaceutical point of view.

Finally, the present invention relates to the therapeutic or prophylactic use of a recombinant viral vector particle obtained by a procedure according to the invention or of a eukaryotic host cell according to the invention for the preparation of a medicament intended for the treatment of the human or animal body and, preferentially, by gene therapy. According to a first possibility, the medicament can be administered directly in vivo (for example in an accessible tumor, in the lungs by aerosol . . . ). It is likewise possible to adopt the ex vivo approach which consists in taking cells from the patient (stem cells of the bone marrow, peripheral blood lymphocytes, muscle cells . . . ), in transfecting or infecting them in vitro according to the techniques of the art and in readministering them to the patient.

The invention likewise extends to a method of treatment according to which a therapeutically efficacious quantity of a recombinant viral vector particle obtained by a procedure according to the invention or of a eukaryotic host cell according to the invention is administered to a patient having need of such a treatment.

The present invention is described more completely with reference to the following figures and with the aid of the following examples.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the genome of the human adenovirus of type 5 (represented in arbitrary units from 0 to 100), indicating the position of the different genes.

FIG. 2 is a schematic representation of the pTG4701 vector in which the left 5' region of the 5 adenovirus is modified by the insertion of LoxP direct repetitions on both sides (in positions 161 and 460) of the encapsidation region (psi).

FIG. 3 is a schematic representation of the pTG8595 vector with the E1, E3 and E4 regions and comprising an expression cassette of the LacZ gene controlled by the MLP promoter and a polyA sequence (pA).

FIG. 4 is a schematic representation of the pTG4707 vector with the E1, E3 and E4 regions and comprising two LoxP direct repetitions on both sides of the psi encapsidation region.

FIG. 5 is a schematic representation of the pTG4662 vector, a recombinant adenoviral vector with the E1 and E3 regions and carrying the same LacZ cassette as pTG8595.

FIG. 6 is a schematic representation of the vector pTG4667, a recombinant adenoviral vector with the E1, E3 regions and the ORFs 1 to 4 of E4 deleted and carrying the same LacZ cassette as pTG8595.

FIG. 7 is a schematic representation of the pTG4702 vector which derives from the above vector by insertion of an expression cassette of the CRE-ER hybrid protein (indicated cre and hER) in place of that of LacZ.

#### EXAMPLES

The following examples only illustrate one method of carrying out the present invention.

The constructs described below are carried out according to the general techniques of gene therapy and of molecular cloning, detailed in Maniatis et al., (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, N.Y.) or according to the recommendations of the manufacturer when using a commercial kit. The cloning steps employing bacterial plasmids are carried out in the strain *Escherichia coli* (*E. coli*) 5K (Hubacek and Glover, 1970, J. Mol. Biol. 50, 111-127) or BJ5183 (Hanahan, 1983, J. Mol. Biol. 166, 557-580). This latter strain is preferentially used for the homologous recombination steps. The techniques of amplification by PCR are known to the person skilled in the art (see, for example, PCR Protocols—A guide to methods and applications, 1990, edited by Innis, Gelfand, Sninsky and White, Academic Press Inc.). Being a question of the repair of restriction sites, the technique employed consists in filling the protruding 5' ends with the aid of the large fragment of DNA polymerase I from *E. coli* (Klenow).

As far as the cellular biology is concerned, the cells are transfected according to the standard techniques well known to the person skilled in the art. The calcium phosphate technique can be mentioned (Maniatis et al., supra), but any other protocol can likewise be employed, such as the DEAE dextran technique, electroporation, methods based on osmotic shock, microinjection of a selected cell or methods based on the use of liposomes. As for the culture conditions, they are conventional except when specified.

In the examples which follow, recourse is had to the following cell lines:

Line 293 derived from embryonic human kidney (Graham et al., 1977, supra, which results from the integration into its chromosomes of the 5' end of the genome of Ad5 (ITR5', encapsidation sequence and E1 region) (available at the ATCC under reference 1573).

Line TG1653 (described in the International Application WO94/28152, Example 8) which derives from the line 293 transformed in a stable manner by the plasmid pTG1653 carrying the E4 region of Ad5 (nt 32800 to 35826) and the expression cassette of the pac (Puromycin Acetyl Transferase) puromycin resistance gene (Morgenstern and Land, 1990, Nucleic Acids Res. 18, 3587-3596).

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It is understood that other cell lines can be used.

In addition, the fragments of adenoviral genome employed in the different constructs described below are indicated precisely according to their positions in the nucleotide sequence of the genome of Ad5 such as disclosed in the Genebank databank under the reference M73260.

## Example 1

## Formation of a Helper Virus

This example describes the construction of an adenoviral helper virus defective for the functions E1 and E4 and comprising an loxP recombination sequence positioned on both sides of the encapsidation region.

The loxP sequence is carried by the oligonucleotides oTG6374 and oTG6522 (SEQ ID NO: 4 and 5). These are reassociated and then introduced into the HindIII site of the p poly II vector (Lathe et al., Gene 57, 193–201) to give pTG4691.

The vector pTG8343 arises from the insertion in the p poly II vector of a part of the 5' end of the adenoviral genome, namely the sequences extending from the nucleotides (nt) 1 to 458 and 3323 to 5788. This is linearized by Sall and ligated with the Sall-XhoI fragment isolated from pTG4691 carrying the loxP sequence. pTG4695 is obtained, which comprises an loxP sequence in position 450 of the adenoviral genome or at 3' of the encapsidation region. A second loxP sequence is cloned in the form of a SmaI-PvuII fragment purified of pTG4691 in the preceding vector linearized by AflIII (position 161) and treated with Klenow. The pTG4701 vector thus obtained comprises two direct loxP repetitions surrounding the psi encapsidation region (FIG. 2).

The modified encapsidation region is exchanged for its genomic homolog by the technique of homologous recombination. To this end, the *E. coli* strain is co-transformed with the BglII fragment of pTG4701 and the pTG8595 vector (FIG. 3) digested by ClaI. The latter is a recombinant adenoviral vector with the E1 (nt 459–3328), E3 (nt 28592–30470) and E4 (nt 32800–35826) regions deleted and comprising an expression cassette of the LacZ gene under the control of the MLP promoter in place of the E1 region. The recombined vector is designated pTG4707 (FIG. 4). After transfection in the TG1653 complementation line (E1<sup>+</sup>, E4<sup>+</sup>) and amplification, the Ad TG4707 viruses are purified on a cesium chloride gradient so as to form a viral stock titrating approximately 2×10<sup>8</sup> pfu/ml.

## Example 2

Construction of a Recombinant Adenoviral Vector  
Producing a Recombinase

This example describes the construction of an adenoviral vector expressing the CRE-ER hybrid protein. In the absence of estrogen, the latter is produced in an inactive form but in the presence of the hormone, it adopts an active conformation. The adenoviral vector is devoid of most of the E1 and E3 regions and of the part of the E4 region other than the ORFs 6 and 7 (Open Reading Frame). The expression of these two genes is sufficient to assure the E4 function without necessity for complementation (Ketner et al., 1989, Nucleic Acids Res. 17, 3037–3048). Two types of vectors have been constructed. In the first (pTG4708), the CRE-ER gene is placed under the control of the SV40 promoter although in the second (pTG5630), it is directed by the CMV promoter.

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In the first place, the pTG653 vector carrying the E4 region of Ad5 (nt 32800 to 35826; see WO 94/28152), is digested by AvrII and BglII and then treated by Klenow before being religated on itself. pTG4660 which carries an expression cassette of the ORFs 6 and 7 under the control of the homologous promoter E4 is obtained. This is introduced into an adenoviral vector by homologous recombination. To this end, the vector pTG4662 (FIG. 5) is chosen which comprises the ITR 5' and the encapsidation sequences (nt 1 to 458), an expression cassette of the LacZ gene in the place of the E1 region and the remaining adenoviral sequences with the E3 region (nt 3329 to 27870 and 30749 to 35935) deleted. The *E. coli* BJ strain is co-transformed by the FspI-MunI fragment descended from pTG4660 and pTG4662 digested by SmaI. This recombination event allows pTG4667 (FIG. 6) to be generated, in which the cassette ORFs 6 and 7 replace the E4 wild region.

## A. Construction of the pTG4708 Vector

In parallel, the expression cassette of the CRE-ER hybrid recombinase is isolated from the pCre-ER vector (Metzger et al., 1995, supra) in the form of a Sall fragment and cloned in the vector pTG8343 previously linearized by this same enzyme. pTG4699 and pTG4700 which differ by the orientation of the cassette are obtained.

The vectors pTG4702 (FIG. 7) and pTG4703 are obtained by homologous recombination between a purified SgrAI-BstEII fragment of pTG4699 and pTG4700 respectively and the adenoviral vector pTG4667 linearized by ClaI. They carry the CRE-ER expression cassette (different orientation for each of them) in place of the E1 region and are devoid of the E3 region and of the ORFs 1 to 4 of E4. They are transfected in the 293 and 1653 cells so as to ensure that the expression of the recombinase is not toxic to cell growth and to viral multiplication.

The construction of an adenoviral vector, in addition, defective for the E2 region can be carried out starting from previous constructs by treatment with the AscI enzyme and religation. The clones are isolated which have reintegrated the AscI fragment but in opposite orientation with respect to the parent vector. The candidates can be determined by simple enzymatic digestion with enzymes whose sites are present on the fragment, for example BamHI and are designated pTG4708. The aim of this reversal of the orientation is to interrupt the transcription units coding for E2A, the polymerase and the hexon and to render the E2 function defective. It is equally possible to introduce deletions of all or part of the E2 region.

## B. Construction of the pTG5630 Vector

The vector pTG4667 is digested by the SnaBI enzyme and then religated. The vector pTG5613 similar to pTG4667 is selected except for the reversal of the adenoviral fragment SnaBI (positions 10307 to 25173) covering a large part of the coding sequences of the E2 region. This reversal allows a defective virus to be generated which is incapable of producing the functional expression products of E2.

In addition, the sequences coding for CRE-ER are isolated by EcoRI digestion and treated by Klenow before being introduced upstream of the CMV promoter, giving rise to pTG5625. The vector pTG5630 is obtained by homologous recombination between the PacI-BstEII fragment isolated from pTG5625 and the vector pTG5617 linearized by ClaI. pTG5630 is defective for the E1 and E2 functions, with the major part of E3 deleted, and carries the "CMV—CRE-ER promoter" cassette in place of E1 in reverse orientation with respect to the 5' ITR.

## Example 3

## Procedure for Preparation of Viral Particles of an Adenoviral Vector by the System CRE and loxP

## A. Employment of the Vector pTG4708 and the Helper pTG4707

In this example, the following are used:

- (1) an adenoviral vector (pTG4708) defective for all of the adenoviral functions with the exception of E4 and carrying the sequences coding for the recombinase CRE-ER, a helper virus (pTG4707) defective for the functions E1, E3 and E4 and containing two loxP direct repetitions placed on both sides of the encapsidation region, and the 293 complementation cell line complementing the E1 function.

The vectors pTG4707 and pTG4708 are co-transfected in 293 cells and cultured at first in a conventional medium not containing estrogen. The viral particles can be formed in the cells which comprise the two vectors since they are mutually complementary. In fact, the E2 proteins are produced from the helper virus, the E4 proteins from the adenoviral vector and the E1 proteins are supplied by the cell line. In addition, the CRE-ER hybrid protein is produced in its inactive form since the culture medium is not supplemented by hormone such that the genome of the helper virus is able to be encapsidated. The plaques thus produced contain a mixed population of virus, one part containing the genome of the adenoviral vector and the other part that of the helper virus. This step allows an amplification of the quantity of virus with the aim of improving the titers.

The step of selective production of the particles of adenoviral vector is carried out by introducing into the culture medium estradiol according to the conditions detailed in Metzger et al. (1995, supra). The presence of estradiol will allow the CRE-ER recombinase to be activated which, once functional, is able to induce a recombination event between the loxP sequences. The viruses generated under these conditions are purified, the DNA is isolated and the deletion of the encapsidation region verified either by enzymatic digestion or hybridization with an appropriate probe complementary to that according to the technology of Southern.

## B. Employment of the Vector pTG5630 and of the Helper pTG4707

2.5 to 5  $\mu$ g of pTG5630 vector are transfected in the 293 line under the conventional conditions. The following day the transfected cells are superinfected by the helper virus AdTG4707 at a rate of approximately 0.04 pfu/cell and cultured in a DMEM medium depleted of Phenol Red (the latter being capable of having an estrogenic activity). Two conditions for culture were studied in parallel: medium supplemented by  $\gamma$ -estradiol (Sigma; E4389) at a concentration of  $10^{-6}$  and  $10^{-7}$  M after the third passage and nonsupplemented medium. The cell culture is harvested at 4 days post-infection and a part of the harvest amplified by successive passages over fresh 293 cells. The other part is preserved for the viral DNA analyses. A significant difference is observed in the state of the cells according to the culture conditions. In fact, the cytopathy is less pronounced in the presence of estradiol than in its absence, this counting from the third passage, which allows a difference to be shown at the level of viral production.

In order to verify this point, the DNA of the viruses produced during the four first passages is isolated from an identical volume of culture, digested by the enzyme MunI and analyzed by Southern with the aid of a radioactive probe complementary to a sequence upstream of the E4 region. After hybridization, an MunI fragment of 1.7 kb is visual-

ized in the case of the viruses descended from the pTG5630 vector and a MunI fragment of 1.1 kb in the case of the AdTG4707 helper viruses. These conditions allow the relative quantity of virus of interest and helpers generated in each amplification cycle to be seen.

Under the conditions where the CRE-ER recombinase is inactive (in the absence of estrogen), a simultaneous amplification of the two types of virus is observed showing itself by a more and more intense signal in proportion to the passages. On the other hand, when the culture medium is supplemented by estradiol, the signal corresponding to the helper virus decreases although that specific for AdTG5630 increases with each passage. These results indicate that the activation of the recombinase is accompanied by a preferential amplification of the virus of interest.

The excision of the encapsidation region of the helper virus is demonstrated by Southern analysis on the viral DNA preparations digested by the enzyme AflII and employing a specific probe of the encapsidation region hybridizing to a fragment of 3.7 kb in the case of the AdTG5630 virus and to a fragment of either 800 bp in the case of the complete helper virus or 400 bp in the case where the encapsidation sequences bordered by the loxP sites are excised.

The intensity of the signal corresponding to the fragment of 3.7 kb increases in proportion to amplification cycles whatever the culture conditions although that corresponding to the 800 bp fragment only increases in the absence of estradiol. On the other hand, in the presence of the hormone, the signal corresponding to the helper virus grows weaker. With high exposure, it is possible to demonstrate a band at 400 pb indicating that the encapsidation region of the helper viruses is excised by the action of the recombinase.

In their entirety, these results show that the CRE-LoxP system can be adapted to the adenoviruses to reduce the contamination of the adenoviral preparations by the helper virus.

## Example 4

## Formation of a Stable Line Expressing the CRE-ER Hybrid Recombinase

The pCre-ER vector is transfected, in a conventional manner, in the 293 cells at the same as a selection vector (for example pRC-CMV conferring resistance to commercially available neomycin (Invitrogen), pTG1643 conferring resistance to puromycin described in WO 94/28156). After transfection, the cells are cultured in selective medium containing the antibiotic and the resistant clones (designated 293/CRE-ER) are isolated and are tested for their capacity to produce an adenoviral vector.

The same technology is used to generate a 1653 line (complementing the adenoviral functions E1 and E4) expressing the CRE-ER gene product.

The 293/CRE-ER cells thus generated are co-transfected by the pTG4707 helper virus and a recombinant adenoviral vector defective for all the functions with the exception of E4 (such as those described in WO 94/28152) and then cultured in a conventional medium so as to generate a mixed and amplified population of viral particles. After a certain time, the culture medium is replaced by a medium containing estradiol in order to produce the recombinase in its active form and to prevent the production of AdTG4707 viral particles.

The 1653/CRE-ER line will be useful for the preparation of vectors defective for all of the essential functions (see WO 94/28156). The technology employed is comparable to

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that described above, namely: co-transfection by the pTG4707 helper virus and a minimum recombinant adenoviral vector, culture in selective medium which is not supplemented by estradiol for a sufficient time and then addition of the hormone to the culture medium and recovery of the viral particles produced.

What is claimed is:

1. A process for preparing an adenoviral particle comprising a recombinant adenoviral vector, which comprises:

(i) preparing a recombinant adenoviral vector deficient for replication;

(ii) preparing a helper vector which complements in trans said recombinant adenoviral vector deficient for replication and which comprises a first recombination sequence 5' and a second recombination sequence 3' of a region essential to the propagation of said helper vector, wherein said recombination sequences are recognized by a recombinase;

(iii) introducing the recombinant adenoviral vector and the helper vector into a permissive or an appropriate cell line wherein said cell line does not constitutively produce active recombinase;

(iv) culturing said introduced cell line under appropriate conditions to increase the quantities of said helper vector and of said recombinant adenoviral vector;

(v) culturing said introduced cell line under appropriate conditions to allow the production of the viral particle in the presence of an active recombinase able to recognize said first and second recombination sequences; and

(vi) recovering the adenoviral particle in the cell culture; wherein said helper vector, said recombinant adenoviral vector, or said cell line comprises a DNA fragment coding for a recombinase and elements for its expression; and wherein said elements confer an inducible expression in response to an inducer, or said recombinase is expressed in an inactive form and is activated in the presence of a ligand, or both.

2. The process of claim 1, wherein said region essential to the propagation of said helper vector is the encapsidation region.

3. The process of claim 1, wherein said helper vector comprises ITRs 5' and 3', an encapsidation region and at least one viral gene selected from the group consisting of E1, E2, E4 and L1-L5 regions of an adenovirus genome.

4. The process of claim 1, wherein said recombinant adenoviral vector deficient for replication is defective for the E1 and/or E4 functions.

5. The process of claim 1, wherein said recombinant adenoviral vector deficient for replication is defective for all of the adenoviral functions, except the left and right ITR regions and the packaging region.

6. The process of claim 1, wherein said recombinant adenoviral vector deficient for replication is defective for all of the adenoviral functions with the exception of E4 functions.

7. The process of claim 1, wherein said recombinase is selected from the group consisting of CRE, FLP and R.

8. The process of claim 1, wherein said recombinase is a hybrid protein resulting from the fusion of a polypeptide sequence having a recombinase activity with a linking region which can be recognized by a ligand.

9. The process of claim 1, wherein said cell line is a cell line complementing an adenoviral vector deficient for replication.

10. The process of claim 1, wherein said cell line comprises a DNA fragment, coding for a recombinase which is

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a hybrid protein resulting from the fusion of a polypeptide sequence having a recombinase activity with a linking region which can be recognized by a ligand.

11. The process of claim 1, wherein said first and second recombination sequences are in the same orientation.

12. The process of claim 1, wherein said first and second recombination sequences are in the reverse orientation.

13. The process of claim 1, wherein said first and second recombination sequences are selected from the group consisting of loxP, FRT and R.

14. The process of claim 1, wherein the inducer is added in step (vi).

15. The process of claim 1, wherein the recombinant adenoviral vector and the helper vector are co-introduced into said permissive cell line or an appropriate cell line in step (iii).

16. The process of claim 1, wherein said helper vector is deficient for replication.

17. The process of claim 2, wherein said encapsidation region is attenuated.

18. The process of claim 17, wherein said attenuation is obtained by deletion of a part of said encapsidation region.

19. The process of claim 16, wherein said helper vector comprises an adenovirus genome devoid of all or part of E1 and/or E4 regions.

20. The process of claim 4, wherein said recombinant adenoviral vector deficient for replication lacks ORFs 1 to 4 of E4 region.

21. The process of claim 8, wherein said recombinase is inactive in the absence of said ligand.

22. The process of claim 8, wherein said polypeptide sequence having a recombinase activity is CRE.

23. The process of claim 8, wherein said linking region is the linking region of the human estrogen ER.

24. The process of claim 8, wherein said recombinase is a hybrid recombinase CRE-ER, said first and second recombination sequences are lox and said ligand is estradiol.

25. The process of claim 8, wherein the ligand of the linking region is added in step (vi).

26. The process of claim 9, wherein said cell line is a 293 cell line.

27. The process of claim 9, wherein said cell line is a cell line complementing an E1 and/or E4 function of said adenoviral vector defective for replication.

28. The process of claim 10, wherein said recombinase is inactive in the absence of said ligand.

29. The process of claim 10, wherein said polypeptide sequence having a recombinase activity is CRE.

30. The process of claim 10, wherein said linking region is the linking region of the human estrogen ER.

31. The process of claim 10, wherein the ligand of the linking region is added in step (vi).

32. A process for preparing an adenoviral particle comprising a recombinant adenoviral vector, which comprises:

(i) preparing a recombinant adenoviral vector deficient for replication;

(ii) preparing a helper vector which complements in trans said recombinant adenoviral vector deficient for replication and which comprises a first recombination sequence 5' and a second recombination sequence 3' of a region essential to the propagation of said helper vector, wherein said recombination sequences are recognized by a recombinase;

(iii) introducing the recombinant adenoviral vector and the helper vector into a permissive or an appropriate cell line wherein said cell line does not constitutively produce active recombinase;

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- (iv) culturing said introduced cell line under appropriate conditions to increase the quantities of said helper vector and of said recombinant adenoviral vector;
  - (v) culturing said introduced cell line under appropriate conditions to allow the production of the viral particle in the presence of an active recombinase able to recognize said first and second recombination sequences; and
  - (vi) recovering the adenoviral particle in the cell culture; wherein said recombinase is added to the cell culture in step (v).
33. The process of claim 32, wherein said recombinase is added in substantially pure form.
34. A process for preparing an adenoviral particle comprising a recombinant adenoviral vector, which comprises:
- (i) preparing a recombinant adenoviral vector deficient for replication;
  - (ii) preparing a helper vector which complements in trans said recombinant adenoviral vector deficient for replication and which comprises a first recombination sequence 5' and a second recombination sequence 3' of

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- a region essential to the propagation of said helper vector, wherein said recombination sequences are recognized by a recombinase;
- (iii) introducing the recombinant adenoviral vector and the helper vector into a permissive or an appropriate cell line wherein said cell line does not constitutively produce active recombinase;
- (iv) culturing said introduced cell line under appropriate conditions to increase the quantities of said helper vector and of said recombinant adenoviral vector;
- (v) culturing said introduced cell line under appropriate conditions to allow the production of the viral particle in the presence of an active recombinase able to recognize said first and second recombination sequences; and
- (vi) recovering the adenoviral particle in the cell culture; wherein the ratio of viral particles comprising a recombinant adenoviral vector to those containing the helper vector in the cell culture is greater than 50%.

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## Review

# Inactivation of gene expression in plants as a consequence of specific sequence duplication

R. B. Flavell

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom

**ABSTRACT** Numerous examples now exist in plants where the insertion of multiple copies of a transgene leads to loss of expression of some or all copies of the transgene. Where the transgene contains sequences homologous to an endogenous gene, expression of both transgene and endogenous gene is sometimes found to be impaired. Several examples of these phenomena displaying different features are reviewed. Possible explanations for the observed phenomena are outlined, drawing on known cellular processes in *Drosophila*, fungi, and mammals as well as plants. It is hypothesized that duplicated sequences can, under certain circumstances, become involved in cycles of hybrid chromatin formation or other processes that generate the potential for modification of inherited chromatin structure and cytosine methylation patterns. These epigenetic changes could lead to altered transcription rates or altered efficiencies of mRNA maturation and export from the nucleus. Where the loss of gene expression is posttranscriptional, antisense RNA could be formed on accumulated, inefficiently processed RNAs by an RNA-dependent RNA polymerase or from a chromosomal promoter and cause the observed loss of homologous mRNAs and possibly the modification of homologous genes. It is suggested that the mechanisms evolved to help silence the many copies of transposable elements in plants. Multicopy genes that are part of the normal gene catalog of a plant species must have evolved to avoid these silencing mechanisms or their consequences.

questions for all those wishing to exploit transgenic plants in research laboratories, industries, or agriculture; and (iii) they have opened up avenues of research in plant biology, including the means of making mutants.

The series of reports that established the phenomenon were published from 1989 to 1991, some 7 years after the first series of publications announcing the production of transgenic plants (but see refs. 1 and 2). Prominent early publications were those that showed that insertion of an additional copy of a chalcone synthase or dihydroflavonol-4-reductase gene into petunia plants led to the silencing in many but not all the transgenic plants of the inserted gene and its endogenous homologues (3-5). The coordinate silencing of the transgene and the homologous endogenous gene gave rise to the term "cosuppression" (3, 5).

Chalcone synthase facilitates the conversion of coumaroyl CoA and 3-malonyl CoA to chalcone in the pathway of anthocyanin pigment biosynthesis. One gene of the small multigene family of chalcone synthase in petunia is especially active in petunia flowers, where pigment production in the corolla and anthers is substantial. After insertion of the chalcone synthase gene under the control of the "constitutive" cauliflower mosaic virus (CaMV) 35S promoter, up to 50% of the transgenic plants with the new gene showed sectors of reduced or no anthocyanin pigment in the flowers, and in some plants the flowers were completely white (3). This lack of pigment is correlated with very low levels of mRNA from the newly inserted and the endogenous copies of the chalcone synthase genes. In a similar but not identical series of plants Mol and coworkers (6, 7) found from nuclear "run on" experiments that nascent RNA transcription is unaffected. I have confirmed this (unpublished data).

In some plants whose flowers are white due to the inserted chalcone synthase gene, occasional branches occur with purple flowers or flowers with purple sectors. All the flowers on such a branch are usually very similar in pattern compared with the flowers on other branches of the plant. Thus, it can be concluded that a somatically inherited change of

state of the transgene has occurred in this branch and, in some cases, during formation of the meristem of the branch. The occurrence of such epigenetic changes during plant or flower development emphasizes that the silencing phenomenon is reversible and can be under developmental control (5, 8). The new phenotype of a flower (and of the branch containing the flower) can be inherited (9) through sexual reproduction and seed development/germination. Many different "states" of transgene activity in cosuppression are possible based on the variety of flower pigmentation patterns arising in isogenic progeny. The patterns of floral pigmentation imply that the capacity of the transgene to cause suppression is subject to developmental influences.

This example illustrates many of the features of cosuppression but many other examples are now known in six plant species where gene inactivation results from the introduction of homologous sequences (reviewed in refs. 10 and 11). Some of these are noted below.

Tomato plants transformed with a truncated gene encoding part of polygalacturonase under the control of the CaMV 35S promoter showed only low levels of endogenous polygalacturonase mRNA in ripening fruit where it is normally highly induced. Transcripts of the transgene were similarly reduced but only in fruit (12). Thus, transcription of both genes may be essential for mutual inhibition. Degraded RNA products from both genes were found in cosuppressed fruit, suggesting that RNA transcription is not inhibited and therefore loss of mature mRNA is due to posttranscriptional turnover.

Multiple copies of transgenes can interact to cosuppress each other (13, 14). Some *Arabidopsis thaliana* plants carrying multiple closely linked copies of the hygromycin phosphotransferase gene lost resistance to hygromycin during development (14). This multicopy locus also suppressed active copies of the same gene at another locus introduced by crossing two strains. The cosuppression

Frequently, when unexpected phenomena are observed they are ignored, then timidly explored and discussed, and only later published and debated with firm conviction in a more coherent framework. We have now entered the third phase for the unexpected phenomena associated with the silencing of multiple copies of genes inserted into plants and the silencing of endogenous plant genes with sequence homology to the newly inserted DNA. The discoveries are extremely important because (i) they reveal previously unrecognized facets of the control of gene expression except as rare isolated instances; (ii) they raise serious

Abbreviations: CaMV, cauliflower mosaic virus; SAR, scaffold attachment region.

could be reversed by separating the loci by outcrossing. These results suggested that the initial silencing of the multiple inserts at one locus, as well as the silencing/activation of the genes in different loci, is the consequence of specific sequence-homology-dependent interactions.

Similar results were found when a portion of the nopaline synthase gene under the control of the CaMV 35S promoter was introduced into tobacco plants already containing wild-type nopaline synthase genes (15). All progeny containing both introduced genes expressed no nopaline synthase activity. The lack of nopaline synthase activity was dependent on the presence of the portion of the nopaline synthase gene in the genes introduced second. When the wild-type nopaline synthase gene was segregated away from the duplicated nopaline synthase sequence its activity was fully or partially restored in most plants. However, suppression of activity was not correlated with methylation of cytosines in the promoter as found in other cases (2, 16).

Vaucheret (17) has described an example of a transgene inserted into tobacco that contains a chimeric gene of nitrite reductase in the antisense direction under the control of the CaMV 35S promoter linked to a sequence encoding neomycin phosphotransferase under the control of the CaMV 19S promoter. No expression of either gene could be detected and this transgene complex suppresses any gene under the control of the 19S and 35S promoter inserted by sexual crossing. Thus, the transgene complex is a strong cosuppressor of genes containing related sequences. Cosuppression was stable but not observed until transient expression of newly introduced transferred DNA (T-DNA) had subsided—i.e., cosuppression probably is a postintegration event (but see ref. 16). Ninety base pairs of homology in promoter sequences were sufficient to create a cosuppressed condition.

These kinds of results help explain and were reinforced by other examples in which transgenes present in one copy in a plant were much more active than transgenes present in two or more copies (1, 16, 18–21).

In another well-investigated example, transgenic tobacco plants were created that contained two introduced genes, each conferring resistance to a different antibiotic (2, 22–25). The coding sequences of the two genes were unrelated in sequence but they had a common promoter contained in two copies of 300 bp taken from the nopaline synthase gene of *Agrobacterium tumefaciens*. One gene was introduced at the first transformation step and the other was introduced at a second transformation step. Double transform-

ants were produced readily but, surprisingly, in 15% of the double transformants the phenotype of the gene conferring kanamycin resistance (introduced first) was lost after introduction of the second gene conferring hygromycin resistance. In this case though, in contrast to the previously mentioned examples, there was no cosuppression in that the plants that had lost kanamycin resistance retained hygromycin resistance. (This could have been the result of selection for hygromycin resistance in the second transformation step.) Loss of kanamycin resistance was accompanied by methylation of specific CpG residues in its nopaline synthase promoter sequence. The dependence of the trans-inactivation on the second gene encoding hygromycin resistance was illustrated by at least partial reactivation of the kanamycin-resistance gene and loss of cytosine methylation in its promoter when the hygromycin gene was segregated away from the kanamycin-resistance gene.

When the antibiotic-resistance transgenes were combined by sexual crossing, instead of by transformation, similar inactivation of the kanamycin-resistance phenotype in the presence of the hygromycin-resistance gene was recorded, thus eliminating the possibility that physiological states peculiar to the transformation procedures were the cause of kanamycin gene inactivation. Independently integrated kanamycin-resistant transgenes whose activity could not be suppressed by specific hygromycin-resistance transgenes introduced by a second transformation event also could not be inactivated by the bringing together of the two introduced genes by sexual crossing. Different transgene combinations produced no, partial (unstable), or complete (stable) trans-inactivation of kanamycin resistance. These results imply that the ability to trans-inactivate or to be trans-inactivated is defined by the state of the gene loci, including possibly their position in the chromosome and/or in the nucleus. Of special interest is the observation that in some plants homozygous for kanamycin-resistance genes, complete somatically stable trans-inactivation of kanamycin resistance occurred more readily than in plants heterozygous for the kanamycin-resistance genes (see also ref. 26).

Assessment of all these examples indicates that cosuppression and trans-inactivation of genes are dependent on sequence homology, can be epigenetically reversible, break down to various extents when the homologous loci are segregated away from one another, are sometimes dependent on transcription or a promoter, are sometimes under developmental control, do not occur with all copies of transgenes, and are sometimes associated with changes in the cytosine methylation pattern of genes. In some examples, the inactivation is reciprocal—i.e., all copies

are inactivated while in others only one copy is inactivated. Homozygous transgenes are sometimes silenced more effectively than hemizygous transgenes. Loss of gene expression appears to be due to inhibition of transcription in some cases and degradation of mRNA in others. This plethora of unexpected phenomena needs to be explained.

The examples quoted above have emerged from recent research involving transgenes. However, a few examples of allelic interactions leading to silenced states of endogenous genes, some of which are inherited, have been known in plants for a long time (9–11). These examples include paramutation, in which one special allele (the paramutable allele) is converted to a state of activity displayed by the other allele (the paramutagenic allele). The new state is inherited but is often epigenetically unstable and may revert after the paramutable allele has been separated from the paramutagenic allele in progeny segregation (27–29).

Studies of the *R* locus in maize, which confers pigmentation in the aleurone layer of the seed, have shown that paramutable alleles are inherently unstable even in the absence of a paramutagenic allele. This suggests that gene structure is an important element of the potential to interact with a homologous allele. The developmental stage when the genetic change occurs and the subsequent stability of the paramutated allele also differ from one paramutable allele to another (29). Meyer *et al.* (30) discovered paramutagenic-like versions of the maize *A1* gene inserted into petunia under the control of the CaMV 35S promoter. Epigenetic variants of an unstable transgene were characterized. One homozygous variant had lost *A1* gene expression; both copies were hypermethylated in their promoter region and showed paramutagenic behavior that led to a permanent or temporary inactivation of *A1* gene expression in heterozygotes with other *A1* transgene alleles. This inactivation correlated with hypermethylation of both alleles. The extent of paramutation was variable during plant development. This example then provides one mechanism for trans-inactivation: methylation provoked by a similarly methylated paramutagenic allele.

Another discovery that I believe is very relevant to the topic under review came from molecular analysis of a series of alleles at the *niv* locus in *Antirrhinum* that controls flower pigment production. The series includes closely related semidominant and recessive alleles where the semidominant alleles inactivate wild-type alleles in heterozygotes. Each of the semidominant alleles investigated has inversions and multiple copies of *niv* gene sequences. After consideration of several

models for how alleles with these sequence aberrations might confer dominance Coen *et al.* (31, 32) concluded that the inactivation of transcription probably was a consequence of a physical interaction between the semidominant and wild-type alleles.

The similarities between paramutation, the behavior of semidominant alleles, and the trans-inactivation phenomena seen with transgenes raise the question whether all the phenomena are consequences of the same collection of mechanisms. This reviewer believes they are.

### Hypotheses to Explain the Trans-Inactivation Phenomena

Four categories of explanation have been offered by others to account for the phenomena (5-11, 13, 15, 30, 33-37). They are not mutually exclusive and no one is applicable to all the examples surveyed above. The first hypothesis suggests that the genes involved adopt an epigenetic state that affects gene expression after their physical interaction. In the second hypothesis gene expression is inhibited due to competition between genes for nondiffusible factors, essential for ordered transcription or translation, such as the nuclear matrix or nuclear envelope. The third and fourth hypotheses apply to cases in which transcription is not inhibited but specific mRNA degradation is the cause of loss of gene expression. The hypothesis debated most involves the production of unintended antisense RNA formation and the degradation of mRNA sense-antisense duplexes. The other involves the accumulation of higher levels of a specific RNA due to the addition of extra copies of its gene and the consequential degradation of all of this mRNA species by some unknown mechanism.

Below I outline nuclear processes that I believe provide a useful background to consider these and other explanations for the whole range of trans-inactivation phenomena reviewed above. This outline is then followed by a review of some of the supporting evidence for its constituent elements.

The processes to be considered first in summary and in more detail later are shown in Fig. 1. Current views of chromatin behavior and transcription imply that DNA in condensed chromatin is recruited into a decondensed form and becomes attached to the nuclear matrix, and regulated transcription is initiated. All these processes are programmed and regulated by complex interactions between a large array of regulatory proteins and DNA in chromatin.

If it is hypothesized that under certain conditions homologous DNA sequences interact in somatic nuclei to form a hybrid DNA duplex or triplex, then there could

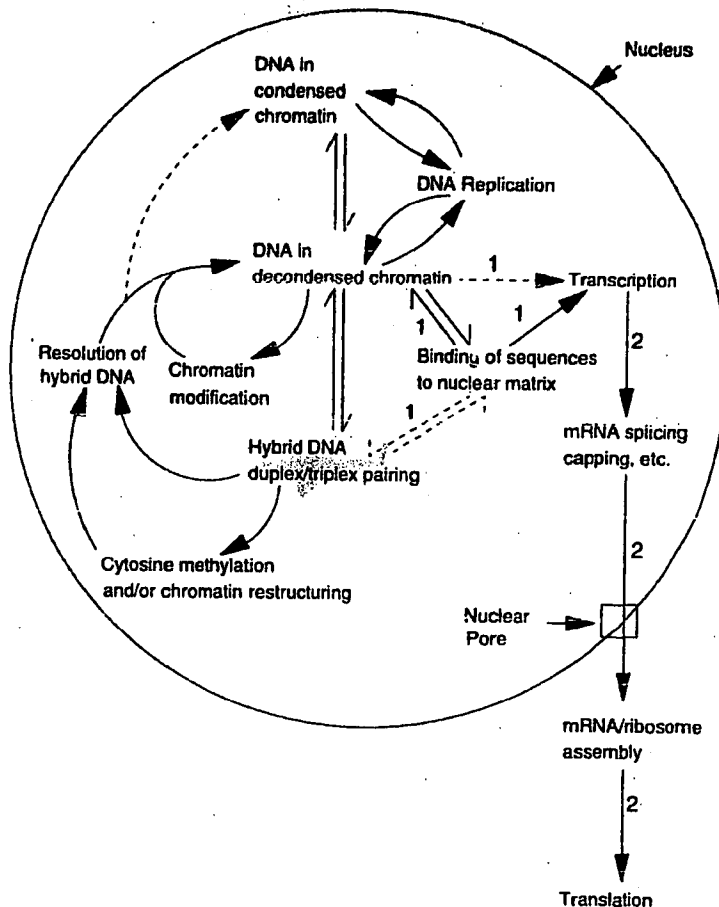


FIG. 1. Schematic illustrating the processes that could suffer aberrations to cause cosuppression and trans-inactivation. Details are described in the text.

be several unusual consequences. First, a new chromatin/DNA structure would result while the hybrid DNA was sustained. Second, there could be an exchange of chromatin proteins to create a new state of the chromatin/genes involved in the duplex. Third, if heteroduplex DNA were established, then different patterns of cytosine methylation could be imprinted into the participating DNA strands. These events should be viewed as being part of a dynamic reiterative cycle, as shown in Fig. 1, where reactions in segments of the cycle can occur many times per cell cycle or only spasmodically in different cells during development. On each occasion, the same or different copies of a sequence could be involved. The outcome of each stage could be different on each occasion, creating instability of allele chromatin structure and variation between alleles or, alternatively, the outcome could be constant, leaving allele chromatin structure very stable and homogeneous.

Any new chromatin state of a gene created via hybrid association or by gain of a protein or methylated cytosines via other routes could influence the subse-

quent chromatin condensation pattern as depicted in Fig. 1, with some states causing the gene to remain in condensed chromatin and silent. Hybrid DNA structures or those of the new chromatin states might interfere with binding of the genes to the nuclear matrix or with subsequent transcription to result in aberrant expression of some or all copies of the genes. The trans-inactivation would therefore result from interference in steps labeled 1 in Fig. 1.

When trans-inactivation is posttranscriptional, two routes can be hypothesized, affecting steps labeled 2 in Fig. 1. It can be envisaged that altered allelic chromatin states are transcribed, but in the wrong segment of the nucleus, and the mRNA-protein complexes are improperly or inefficiently transported through the nucleus, processed, and exported through the nuclear pore. They are consequently degraded. In an elaboration of this model, one could envisage that if mRNA transport processing, export, and possibly translation were inefficient, or excess mRNA accumulated due to aberrantly high levels of transcription, then an RNA-dependent RNA polymerase could synthesize antisense RNA

molecules on the mRNA templates. The antisense RNAs would form complexes with all the homologous sense mRNAs in the cell and the complexes would be degraded before or after export from the nucleus. Alternatively, the RNA-RNA duplexes could be formed in the cytoplasm and prevent translation. The involvement of an RNA-dependent RNA polymerase has been recently postulated by Lindbo *et al.* (38) to explain the loss of transgene mRNA and viral RNA in virus-resistant tobacco plants containing a nuclear transgene encoding tobacco etch virus coat protein and infected with tobacco etch virus.

In the second route, it is envisaged that transgenes become incorporated into chromosomes under the control of promoters that generate antisense RNA to the transgene. The antisense RNA would inhibit RNA survival and translation as described above.

These scenarios could explain changes in the state of certain homologous genes, their imprinting and the stability/instability of new states, failure to be transcribed, or turnover of their mRNA.

The hypotheses have been divided into those that affect transcription and those that are posttranscriptional. These can be linked if a feedback system exists such that an accumulation of primary transcripts or antisense RNAs in the nucleus influences the chromatin or methylation state of a gene to affect its ability to efficiently participate in transcription. If this is the case, then inherited trans-inactivation of genes with sequence homology could occur due to excess on RNA buildup without hybrid DNA formation. The cycles of modification of chromatin structure shown in Fig. 1 without hybrid DNA formation are depicted to include this sort of possibility.

Evidence for the principal steps envisaged in these hypotheses is reviewed next.

**(i) Interactions Between Loci with Homologous DNA Sequences.** For interactions between loci with homologous sequences to occur, the DNA must be accessible. This suggests that the chromatin must be decondensed and DNA strands of the interacting loci be opened up—presumably during transcription, regulatory protein binding, replication, or recombination. This might explain the need for a promoter for trans-inactivation/cosuppression in some cases. Certain DNA structures such as the presence of inverted repeats might provoke alternative forms of genes that facilitate accessibility of DNA (32). Sequence-specific interaction implies hybrid chromatin or DNA duplex or triplex (39) formation, at least transiently, but this would need to be very efficient to account for the frequency of trans-inactivation observed, unless the chromatin/DNA that interacted was her-

itably imprinted. There is little evidence, direct or indirect, for such regular interactions involving all or most accessible chromatin sectors in somatic plant cells.

In yeast, such a DNA homology-searching process has been inferred to account for the equivalent frequencies of allelic and ectopic (in different position in the chromosome) meiotic recombination between homologous sequences (40). In *Neurospora*, duplicated sequences are detected by a homology searching/sensing process in the haploid nuclei of dikaryons before meiosis (41). The duplicated sequences are modified by cytosine methylation and a high proportion of the modified cytosines are substituted by thymidine. This repeat-induced point mutation process leads to destruction of gene function and elimination of such mutant progeny from the population. The DNA pairing process is not dependent on the meiotic chromosome pairing mechanisms (42). In *Ascomolus immersus* artificial gene repeats are also heritably inactivated, premeiotically, by cytosine methylation but other mutations are not introduced (43). DNA sequences can undergo several rounds of pairing so that multiple copies of a sequence can be inactivated sequentially (44). Thus, the pairing machinery does not distinguish between methylated and nonmethylated copies.

These observations on fungi provide a useful basis for considering possible mechanisms of trans-inactivation/cosuppression in plants. They provide a precedent for a process to control the expression of unusual duplicated sequences involving an efficient homology searching/sensing system. In these fungi, hybrid or paired DNA is presumably recognized in premeiotic cells, directly or indirectly, by a *de novo* methylase or a maintenance methylase that operates on hemimethylated DNA. Any parallel in plants would have to occur, presumably, in any somatic cell.

If duplicated sequences involving a transgene recognize each other and form hybrid DNA, even only transiently, how could different states of the transgenes or transgene and endogenous genes emerge and be inherited? There are two sorts of possibilities for this, with precedents in other kinds of organisms: restructuring of chromatin and DNA sequence modification. These processes are reviewed below.

**(ii) Chromatin Restructuring.** The case for modification of interacting loci via chromatin changes is based on observations established in *Drosophila*. Eleven examples have been reviewed recently (45) in which the expression of a gene is influenced by "sensing" the presence of another specific gene after some kind of localized somatic chromosome pairing (46–48). A transcription factor associated with the chromatin of one of the

genes is postulated to interact also with the promoter/enhancer of another chromosome and modify the expression of this second locus (49). However, another model involving transacting regulatory RNAs as mediators of the effects has also been put forward (45). In a second type of interaction, a gene becomes inactivated by assuming a heterochromatic, repressing chromatin structure from its neighboring sequences. It then pairs with a wild-type allele on another chromosome and a mutant phenotype results because the chromatin structure of the wild-type gene is also converted to an inactive, heterochromatic form. This model is based on the ability of chromatin proteins determining heterochromatic condensation to initiate the condensation process, which then proceeds along the chromosome until some interfering components are encountered. When such initiating proteins are transferred to the wild-type gene via close pairing of chromosomes, the heterochromatic structure is imposed upon it also. Such modes of heterochromatic chromatin assembly are dominant to those normally determining the chromatin structure at the wild-type gene locus. When somatic pairing is disrupted in these cases, more normal gene expression ensues. In other cases, mutant phenotypes are enhanced when pairing is disrupted. The same is true for the trans-sensing effects on polytene chromosome puffs. In such cases, a mutant site will puff and accumulate mRNA when paired with its wild-type homologue but not when these chromosomes are desynapsed or remain homozygously paired.

An important issue for cosuppression and trans-inactivation in plants, as noted above, is how new states of the loci involved, however they are created, are stabilized and inherited. Other genetic studies on *Drosophila* have revealed gene products that appear to influence the state of gene activity through organismal development. Paro (50) has described a model that represents a way to imprint on/off transcription status into the higher-order chromatin structure surrounding a gene. He envisages chromatin of early embryos in a neutral state, allowing signals to activate specific gene loci. When the signals are not received, specific proteins interact with cis-regulatory elements of a gene and act as nucleation signals for a kind of heterochromatization, a local state that is clonally inherited through development. A parallel mechanism is envisaged whereby specific proteins would bind to specific loci to keep them open and available to developmentally controlled specific transcription factors.

The programming of transcriptional competence based on organization of chromatin structure is now receiving much attention in yeast and *Drosophila*, following the discovery of numerous

gene products that influence the expression of many genes and that are likely to work via influencing chromatin protein complexes (50–52). In summary, there is growing evidence from *Drosophila* and yeast, where the most detailed molecular genetic studies have been carried out that, in certain examples at least, chromatin states can be transferred between homologously paired chromatin segments, that competence for transcription is determined by chromatin structure, and that some chromatin states can be clonally inherited when not disturbed.

**(iii) DNA Sequence Modification by Methylation.** The second mechanism that can be envisaged to account for the heritable modifications of the duplicated DNA sequences involved in cosuppression and trans-inactivation is methylation of different cytosine residues. This process could occur either in the hybrid DNA or after establishment of a chromatin structure by any route.

It is attractive to consider this process for many reasons: (i) the process occurs in all plant nuclei; (ii) cytosine methylation changes have been observed to correlate with at least some cases of cosuppression or trans-inactivation (e.g., see refs. 16, 23, and 30); (iii) it results in imprinting of DNA, and imprinted patterns are inherited due to the addition of methyl groups to new DNA strands based on the pattern in the old strand (53); (iv) enhanced cytosine methylation at key sites is known to correlate with modified chromatin structure and gene expression (54); and (v) it is known to be part of the mechanism used to silence artificially duplicated genes in fungi (41–43).

If hybrid DNA is formed between sequences of duplicate transgenes or between transgenes and endogenous sequences, then the DNA strands will be hemimethylated at many sites because the cytosine methylation pattern will differ between the two parental sequences. This is because when transgenes are introduced from *Agrobacterium* they are unmethylated and without a plant-determined chromatin structure. The process of cytosine methylation in CpG and CpXpG motifs must involve *de novo* methylation. It is likely that the stabilized pattern will reflect the chromosomal environment where it is inserted and random processes (30). The pattern may take many cell generations to stabilize. For these reasons, different insertions of the same transgene are likely to have different methylation patterns.

There is a very active methylase that recognizes hemimethylated DNA in plants (55) since 80% of the CpG and of the CpXpG sites are methylated and these have to be methylated after every round of replication. Therefore, given hybrid DNA and accessibility of these

sequences to the appropriate methylase, the number of methylated sites in the hybrid DNA is likely to increase. Upon separation of the hybrid DNA strands and their assimilation back into their parent duplexes, hemimethylated parent templates would exist and would be methylated. Thus, after the interaction, both parental genes would be modified in the regions that formed hybrid DNA—generally in the direction of increased cytosine methylation. If the altered methylation pattern affected transcription directly or indirectly by the altered binding of regulatory proteins or by affecting the condensation pattern into a different chromatin conformation, then new heritable states of gene expression would have been created. The paired DNA sequences would have similar changes imprinted and thus might share similar changes in gene expression in many but not all cases. The process is usefully seen as a dynamic cyclical pathway (see Fig. 1) and multiple rounds of the cycle could occur in each cell cycle. To attain a stable state for all copies of a sequence, many cycles would probably be required.

The observed outcome would depend on the original state(s) of the transgene(s), and of the endogenous loci, and the role of specific cytosine residues in controlling levels of transcription, protein binding, and chromatin structure. Thus, gene expression could be reduced, increased, or unchanged.

A prediction of methylation of hemimethylated hybrid DNA is that homologous sequences should accumulate the same cytosine methylation pattern in the same cell lineage. This is testable. However, the pattern of methylated cytosines established could differ following the resolution of hybrid DNA duplexes in different cells, and thus chimeras would be produced, as has been observed (10, 11). The requirement for a transgene to provoke easily recognized cosuppression frequently may be because only newly inserted genes are in different, unregulated states of cytosine methylation and are therefore capable of giving rise to a variation in cytosine methylation and chromatin patterns via hybrid DNA formation.

**(iv) Inhibition of mRNA Processing, Transport, Export, or Translation.** The first product of RNA transcription undergoes a series of complex processing steps including removal of intervening sequences, if present, capping at the 5' end, and polyadenylation at the 3' end to produce the RNA that is transported through the nuclear pore for translation. How these processes occur within the nuclear architecture is not well documented. It has been proposed that RNAs are transported from the site of synthesis to the pore across a solid nuclear substructure distinct from chromatin (56).

Evidence has been obtained that newly synthesized RNAs are associated with the nuclear matrix and pass along defined paths (refs. 57 and 58 but see ref. 59). The complexes that effect splicing may be localized in specific foci so RNAs requiring splicing may pass through such foci (60, 61). Association of mRNA with a splicing complex inhibits export; export is dependent on release from a spliceosome, and data have been reported to show that 5' capping and a correct 3' end are important for export (62).

Knowledge of these mechanisms implies that, for correct gene expression, RNA transcription may need to take place in an appropriate place in the nucleus and RNAs might traverse specific domains to be properly processed for export. If these requirements are not satisfied due to the relocalization of genes after their interaction and modification, then there could be delays in RNA transport and processing that result in degradation of the RNA.

Alternatively, if inefficient processing and transport were to occur, antisense RNA could be generated by using the accumulated RNA as template, because RNA-dependent RNA polymerase occurs in plant cells (63, 64). Its role and cellular location are unknown. The activity is induced after infection with viruses and after wounding. It is capable of making antisense RNA fragments from plant RNA and appears to display little template specificity. This activity, if nuclear, could synthesize antisense RNA in the nucleus. Alternatively, if cytoplasmic, it could synthesize antisense RNAs on exported RNAs present in excess in relation to regulated translation capacity or on RNAs inefficiently translated due to structural defects through aberrant processing. The antisense RNA products could remain cytoplasmic and prevent translation or enter the nucleus to interfere with RNA maturation.

The inactivation of sense mRNA to the transgene by unintended antisense RNA as an explanation for cosuppression has been debated by several authors (10, 11, 33–35). The authors envisaged that the antisense RNA would originate either from an active promoter in the host chromosome initiating transcription in the opposite direction to the transgene or from another gene promoter in the opposite orientation on the inserted T-DNA.

Efficient production of antisense RNA from a chromosome promoter would lead to down-regulation of sequence-specific gene expression and some antisense RNA has been detected in cosuppressed petunia plants (6, 7). However, there are specific pieces of evidence in examples of cosuppression and trans-inactivation that argue strongly against a chromosomal promoter being the source of antisense RNA and the sole component of a model

much about the phenomena described in this review and for their comments on the manuscript.

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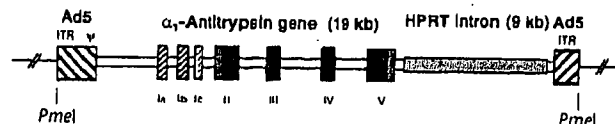
# Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity

Gudrun Schiedner<sup>1,5</sup>, Núria Morral<sup>1,2</sup>, Robin J. Parks<sup>3</sup>, Ying Wu<sup>1</sup>, Suzanne C. Koopmans<sup>1</sup>, Claire Langston<sup>4</sup>, Frank L. Graham<sup>3</sup>, Arthur L. Beaudet<sup>1,2</sup> & Stefan Kochanek<sup>1,5</sup>

Many applications for human gene therapy would be facilitated by high levels and long duration of physiologic gene expression. Adenoviral vectors are frequently used for gene transfer because of their high cellular transduction efficiency *in vitro* and *in vivo*. Expression of viral proteins and the low capacity for foreign DNA limits the clinical application of first- and second-generation adenoviral vectors<sup>1-7</sup>. Adenoviral vectors with all viral coding sequences deleted<sup>8-15</sup> offer the prospect of decreased host immune responses to viral proteins, decreased cellular toxicity of viral proteins and increased capacity to accommodate large regulatory DNA regions. Currently most vectors used *in vivo* for pre-clinical and clinical studies express cDNAs under the control of heterologous eukaryotic or viral promoters. Using an adenoviral vector with all viral coding sequences deleted and containing the complete human  $\alpha_1$ -antitrypsin (*PI*) locus, we observed tissue-specific transcriptional regulation in cell culture and *in vivo*; intravenous injection in mice resulted in high levels of very stable expression for more than ten months and decreased acute and chronic toxicity. These results indicate significant advantages of regulated gene expression using genomic DNA for gene transfer and of adenoviral gene transfer vectors devoid of all viral coding sequences.

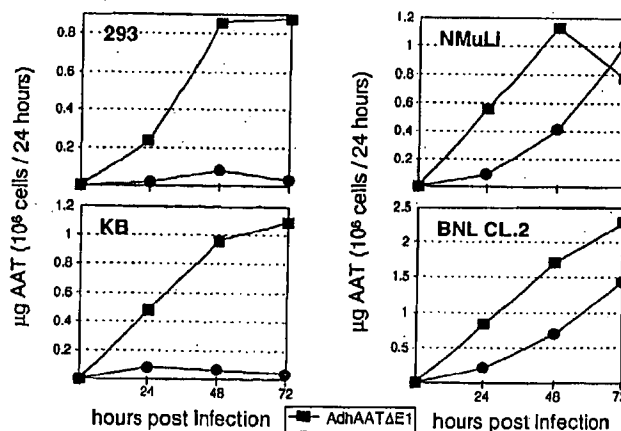
Using the Cre-*loxP* helper-dependent system<sup>15</sup>, we rescued the complete human  $\alpha_1$ -antitrypsin genomic DNA locus (*PI*), including both promoters, all exons and introns, and the polyadenylation signal (a 19-kb DNA fragment) into a high capacity adenoviral vector (AdSTK109; Fig. 1). This DNA fragment was reported to express high levels of  $\alpha_1$ -antitrypsin (AAT) in transgenic mice<sup>16</sup>. A 9-kb intronic DNA fragment from the human hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) gene<sup>17</sup> was included as 'stuffer' DNA to increase packaging efficiency and stability of the vector during production<sup>18</sup>. Thus, the AdSTK109 genome included 28 kb of human genomic DNA and only 0.6 kb of viral noncoding DNA (Fig. 1). AAT, whose major function is to antagonize neutrophilic elastase, is expressed abundantly in hepatocytes and at a lower level in macrophages. Different tissue-specific promoters regulate gene expression in these two cell types<sup>19</sup>.

To test whether adenoviral mediated gene transfer using the *PI* locus resulted in tissue specific expression, cell lines were transduced with either AdSTK109 or AdhAAT $\Delta$ E1, a first generation adenoviral vector that contains a cDNA encoding human  $\alpha_1$ -antitrypsin under control of the murine phosphoglycerate kinase (*Pgk*) housekeeping promoter<sup>6</sup>. Two murine liver cell lines expressed comparable levels of AAT after transduction with both vectors (Fig. 2). However, after transduction of the nonhepatic KB and 293 cell lines, substantial secretion of AAT was observed



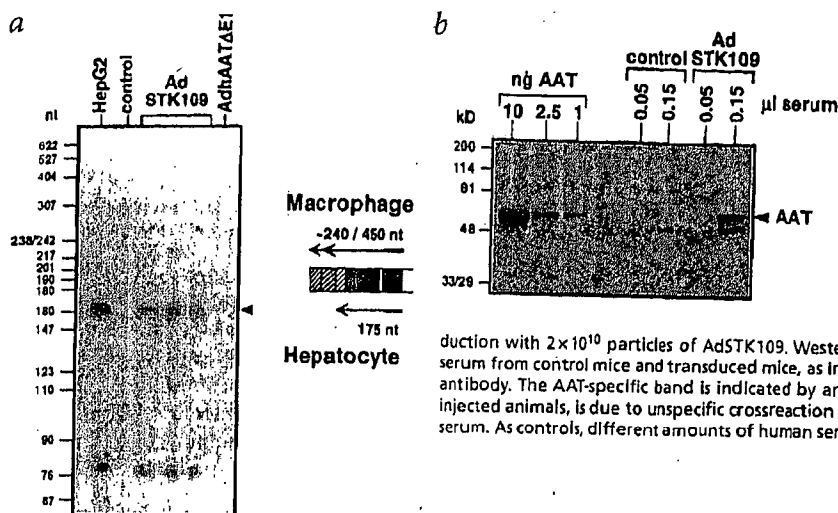
**Fig. 1** Structure of the plasmid pSTK109. pSTK109 has a 28.6-kb insert in the multiple cloning site of pBluescript KSII with, from left to right, the following essential features: the left terminus of adenovirus type 5 (nt 1-440), a 19-kb genomic *SalI* fragment containing human *PI* derived from phage clone  $\alpha$ NN16, a 9-kb intronic DNA fragment derived from human *HPRT* (nt 1777-10609; ref. 17) and the right terminus of adenovirus type 5 (nt 35818-35935). The insert of pSTK109 is flanked by *PmeI* restriction sites; *PmeI* is a rare cutting endonuclease and does not cleave within the insert. The locations of the inverted terminal repeats (ITR) and the packaging signal ( $\Psi$ ) are indicated. *PI* contains 7 exons and 6 introns. The coding sequences are indicated in black, non-coding exons are shaded. The hepatocyte-specific promoter is located between exons Ib and Ic. The macrophage-specific promoter is located upstream of exon Ia.

with AdhAAT $\Delta$ E1 only (Fig. 2), indicating tissue-specific expression of the genomic *PI* fragment in AdSTK109. Similar results, namely liver-specific expression of AdSTK109, were obtained after transduction of two additional nonhepatic cell lines (HeLa-cervix carcinoma; TT-medullary thyroid carcinoma) and Hepala, another murine liver cell line (data not shown). Transduction of the intestinal 407 cell line resulted in expression from both vectors; human intestine is known to express AAT<sup>20</sup>.



**Fig. 2** Expression of AAT *in vitro*. Two human nonhepatic cell lines (293, KB) and 2 murine hepatic cell lines (NMuLi and BNL CL.2), were transduced with a MOI (multiplicity of infection) of 50 with either AdSTK109 or AdhAAT $\Delta$ E1. Secretion of AAT into the cell culture medium was determined by ELISA.

<sup>1</sup>Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. <sup>2</sup>Howard Hughes Medical Institute, One Baylor Plaza, Houston, Texas 77030, USA. <sup>3</sup>Department of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada L8S 4K1. <sup>4</sup>Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. <sup>5</sup>Present address: Center for Molecular Medicine, University of Cologne, Kerpenerstraße 34, 50931 Cologne, Germany. G.S., N.M. & R.J.P. contributed equally to this work. Correspondence should be addressed to S.K. e-mail: stefan.kochanek@medizin.uni-koeln.de



**Fig. 3** Transcriptional initiation and translation after gene transfer *in vivo*. **a**, Primer extension analysis of liver RNA from C57BL/6J mice after transduction with AdSTK109 and AdhAATΔE1. Primer extension analyses were performed using RNA from liver tissues of animals two weeks after tail vein injection of  $2 \times 10^{10}$  particles of either vector or from untreated control animals. RNA from HepG2 cells which constitutively express AAT served as control. The primer hybridizes to exon 2 of *PI* and hepatocyte specific transcription results in a 175-nt product (arrow), whereas macrophage-specific expression would result in transcripts of approximately 240 and 450 nt. **b**, Western-blot analyses of serum from C57BL/6J mice after transduction with  $2 \times 10^{10}$  particles of AdSTK109. Western blots were performed using different amounts of serum from control mice and transduced mice, as indicated, and goat IgG fraction against AAT as primary antibody. The AAT-specific band is indicated by an arrow; the lower band, present both in control and injected animals, is due to unspecific crossreaction of an unknown mouse serum protein to the AAT anti-serum. As controls, different amounts of human serum were used, corresponding to 1, 2.5 and 10 ng AAT.

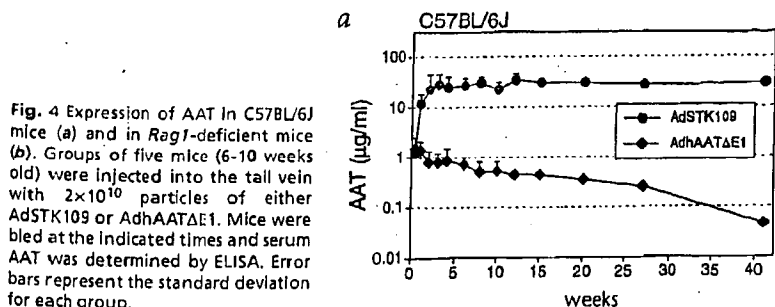
Experiments to evaluate gene transfer *in vivo* were designed to assess the expression potential of the vectors in the absence of host immune responses to the reporter protein<sup>21–24</sup>. C57BL/6J mice were chosen because they do not generate anti-AAT antibodies following intravenous administration of AdhAATΔE1 (ref. 21). Primer extension analysis using liver RNA isolated from AdSTK109-injected animals indicated that only the hepatocyte specific promoter was active in this tissue (Fig. 3a). Northern-blot analysis of liver RNA detected only a single 1.6-kb transcript, demonstrating that the primary transcript was spliced correctly (data not shown). The secreted protein had the correct size (Fig. 3b). Finally, isoelectric focusing (IEF) confirmed that processing of the AAT expressed from AdSTK109 was indistinguishable from that of AAT expressed from AdhAATΔE1 (data not shown). Taken together, after liver transduction with the *PI* locus, the primary transcript was correctly initiated and processed, and the mRNA was correctly translated to yield a properly glycosylated protein.

C57BL/6J mice were transduced with  $2 \times 10^{10}$  particles of either AdSTK109 or AdhAATΔE1 by tail vein injection, and serum levels of AAT were determined by ELISA<sup>21,25</sup> (Fig. 4a). In mice injected with AdhAATΔE1, maximum levels of 2 μg AAT/ml serum were observed 3 days after injection, followed by a slow decline over 10 months to less than 10% of the peak levels. Similar kinetics after adenoviral mediated gene transfer with the *PI* cDNA under control of different promoters were reported previously<sup>7,21,26</sup>. In contrast, in mice that received AdSTK109, AAT levels reached a plateau of approximately 50 μg/ml 3 weeks after injection without decrease during the observation period of 10 months. Injection of AdSTK109 into C3H/HeJ mice also yielded high levels and duration of expression for 10 weeks, the longest period studied to date (data not shown). The persistence of expression in C3H/HeJ mice was unexpected, since these mice produced antibodies to AAT

when a first generation vector was administered<sup>21</sup>. The reasons for the different immune responses to the reporter with the two vectors in C3H/HeJ mice are under investigation.

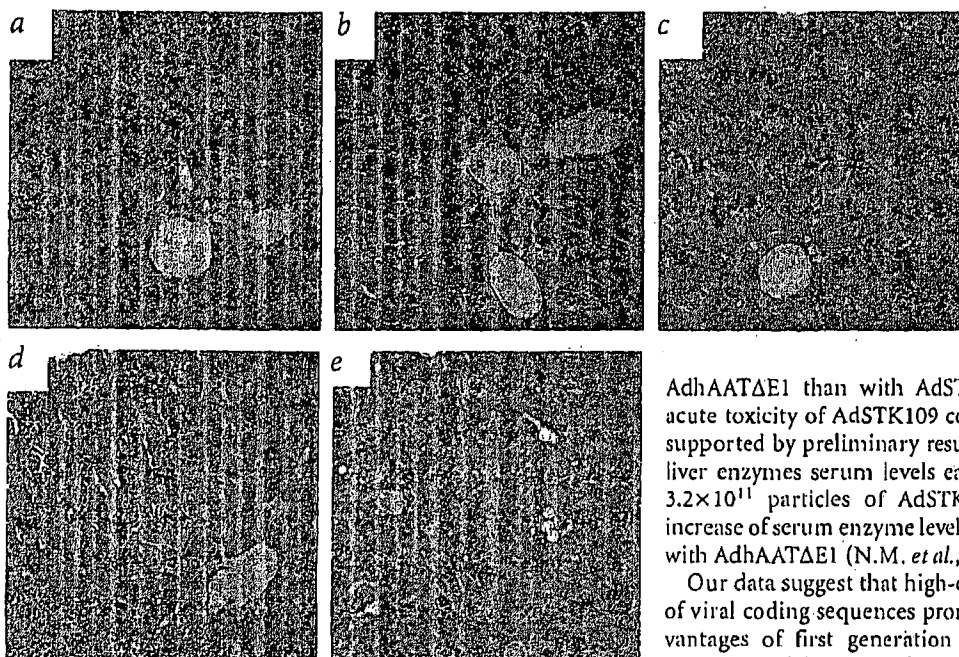
In order to evaluate why expression levels of AAT declined in C57BL/6J mice when injected with AdhAATΔE1 but not with AdSTK109, *Rag1*-immunodeficient mice<sup>27</sup> were injected with either vector (Fig. 4b). The expression profiles for each vector in *Rag1*-deficient mice were similar to results with immunocompetent mice, although the absolute levels of expression were higher (up to 150 μg/ml with AdSTK109). We speculate that the higher expression levels in *Rag1*-deficient versus C57BL/6J mice might be due to absence of nonspecific neutralizing antibodies crossreacting with viral capsid proteins. Compared with immunocompetent mice, higher AAT levels after gene transfer with adenoviral vectors were reported in *scid* mice<sup>7</sup>. The different expression kinetics observed in AdSTK109 versus AdhAATΔE1 injected mice may be a function of the presence of the endogenous genomic promoter or of the *HPRT* intronic 'stuffer' DNA sequence which contains a matrix association region (MAR; ref. 28). Southern-blot analysis indicated that the livers of C57BL/6J mice were transduced with comparable efficiency by AdSTK109 and AdhAATΔE1, with approximately 0.2 to 0.5 copies per cell present 3 days after injection. Data based on groups of 3 animals suggested that in AdhAATΔE1-injected C57BL/6J mice, there was a 65% decline in vector DNA levels between 3 days and 12 weeks; in AdSTK109 injected mice there was a 30% decrease in DNA levels during that period with only a 6% decline between 6 and 12 weeks (data not shown).

Histopathological examination of livers from C57BL/6J mice demonstrated normal morphology between 3 days and 12 weeks after injection with AdSTK109 (Fig. 5). Livers from animals transduced with AdhAATΔE1 were morphologically normal at 3 days and 2 weeks. However, at 6 weeks and more pronounced at



**Fig. 4** Expression of AAT in C57BL/6J mice (**a**) and in *Rag1*-deficient mice (**b**). Groups of five mice (6–10 weeks old) were injected into the tail vein with  $2 \times 10^{10}$  particles of either AdSTK109 or AdhAATΔE1. Mice were bled at the indicated times and serum AAT was determined by ELISA. Error bars represent the standard deviation for each group.





**Fig. 5** Hematoxylin and eosin staining of cross sections of liver tissue from C57BL/6J mice treated as described in Fig. 4. **a**, Control, injected with buffer. **b,c**, Injected with AdSTK109; shown are tissue sections from mice killed 2 weeks (**b**) and 12 weeks (**c**) after injection. **d,e**, Injected with AdhAATΔE1; shown are sections from mice killed 2 weeks (**d**) and 12 weeks (**e**) after injection. Original magnification:  $\times 400$ .

12 weeks, signs of liver injury were apparent with considerable polymorphism of cell bodies and nuclei, scattered necroses and some lipid accumulation (Fig. 5). Unexpectedly, significant lymphocytic infiltrations were not observed, and only sporadic CD4 or CD8 positive lymphocytes were detectable by immunostaining (data not shown). Livers from AdhAATΔE1-injected *Rag1*-deficient mice revealed a biphasic response with hepatocyte proliferation and many mitoses at 3 days, although normal liver morphology was observed at two weeks; at 6 and at 12 weeks, liver injury was apparent that was similar to the pathology observed in AdhAATΔE1 injected C57BL/6J mice at the same points. In contrast, livers of *Rag1*-deficient mice that received AdSTK109 did not show significant abnormalities at these times (data not shown). Although an immune response against viral proteins may have contributed to the abnormal liver histology in C57BL/6J mice late after transduction with AdhAATΔE1, the lack of significant lymphocytic infiltration, and more importantly, the observation of morphologically similar changes in *Rag1*-deficient mice argues that a non-CTL mediated mechanism (for example a direct cytotoxic effect of viral proteins) may explain the liver injury. In C57BL/6J mice, the cell proliferation marker Ki-67 (ref. 29) was present in significantly more cells between 3 days and 6 weeks after injection with AdhAATΔE1 compared with AdSTK109 (data not shown). In *Rag1*-deficient mice, an impressive difference was present at 3 days after injection (Fig. 6). Liver-cell proliferation soon after gene transfer in *Rag1*-deficient mice with AdhAATΔE1 probably reflects increased levels of hepatocyte transduction in immunodeficient versus immunocompetent mice (see above), and higher acute hepatotoxicity with

AdhAATΔE1 than with AdSTK109. The finding of decreased acute toxicity of AdSTK109 compared with AdhAATΔE1 is also supported by preliminary results showing normal ALT and AST liver enzymes serum levels early after intravenous injection of  $3.2 \times 10^{11}$  particles of AdSTK109, but a more than ten-fold increase of serum enzyme levels above normal in animals injected with AdhAATΔE1 (N.M. *et al.*, unpublished).

Our data suggest that high-capacity adenoviral vectors devoid of viral coding sequences promise to overcome the main disadvantages of first generation adenoviral vectors: toxicity and immunogenicity caused by viral gene expression, short duration of expression related to these host responses, and restricted capacity for the accommodation of foreign DNA. Our results contrast with a report of weak and transient expression of AAT using a 'deleted' adenoviral vector with a genome size of only 9 kb in C57BL/6J mice<sup>30</sup>. With the high-capacity vector described here, we achieved efficient hepatic gene transfer and persistent and high levels of expression that were not accompanied by abnormal liver histology. Our results predict that high-capacity adenoviral vectors will be useful for gene transfer and somatic gene therapy because of their improved safety and expression profiles. Their increased capacity for foreign DNA, allowing transfer of multiple expression cassettes, large regulatory elements and even a proportion of genes in their natural genomic context, as demonstrated in this study, is a significant advantage over first and second generation adenoviral vectors.

## Methods

**Mouse strains.** C57BL/6J and *Rag1*-deficient mice were purchased from the Jackson Laboratory. The *Rag1*-deficient mice<sup>27</sup> used in these experiments have C57BL/6J background. All mice were 6–10 weeks old at the time of injection.

**Rescue of AdSTK109.** pSTK109 DNA (10  $\mu$ g; Fig. 1) was cleaved with *PmeI*, followed by phenol extraction and ethanol precipitation. The DNA was transfected into 293 cells that were subsequently infected at a multiplicity of infection (MOI) of 5 with the helper virus AdLC8cluc. After complete cytopathic effect (CPE), the medium and infected cells were harvested and freeze/thawed to release the virus. Aliquots of the crude vector lysate were serially passaged through 293-Cre cells to increase the titre, as previously described<sup>15,18</sup>. The yield of AdSTK109 after CsCl equilibrium

**Fig. 6** Immunostaining of cross sections for the presence of the proliferation marker Ki-67 in liver tissue from *Rag1*-deficient mice 3 days following intravenous injection with AdSTK109 (**a**) and AdhAATΔE1 (**b**). Sections were stained using a rabbit polyclonal antibody against Ki-67 as primary antibody and a biotinylated goat anti-rabbit F(ab) fragment as secondary antibody. Original magnification:  $\times 400$ .



density centrifugation was  $5 \times 10^{11}$  particles produced from  $1.5 \times 10^8$  cells. The infectious titre of AdSTK109 was determined by infecting KB cells in parallel with AdhAATΔE1, using 200 and 1000 particles per cell. DNA isolated from cell nuclei that had been harvested 3 h after infection with either AdSTK109 or AdhAATΔE1 was digested with *SspI* (*SspI* cleaves at nt 341 of Ad 5 DNA) followed by agarose gel electrophoresis and Southern blot analysis. A 440-bp DNA fragment encompassing the left terminus of Ad5 was used as the hybridization probe. Comparison of the signal intensities of the left termini of both vectors allowed the estimation of the infectious titre of AdSTK109 based on the plaque forming unit (pfu) titre of AdhAATΔE1. The particle/infectious units ratio was 20:1 with both vectors. The presence of helper virus in preparations of AdSTK109 was quantitated by performing plaque assays on 293 cells and was found to be 0.1% of the infectious titre of AdSTK109. Thus,  $2 \times 10^{10}$  particles of AdSTK109 (corresponding to  $1 \times 10^9$  infectious units, and the amount that was used for *in vivo* experiments) contained  $1 \times 10^6$  pfu of helper virus. To exclude the presence of replication competent adenovirus (RCA) in the AdSTK109 and AdhAATΔE1 preparations,  $5 \times 10^7$  HeLa cells were infected with  $1 \times 10^{10}$  particles of either vector. The cells were serially passaged for 3 weeks without the appearance of a CPE.

Enzyme-linked immunosorbent assay for AAT. For *in vitro* expression of AAT, nonhepatic and hepatic cell lines were transduced with a MOI of 50 with either AdSTK109 or AdhAATΔE1. The cell culture medium was collected 24, 48 and 72 h after transduction. For *in vivo* expression of AAT in C57BL/6J mice and in *Rag1*-deficient mice, groups of 5 mice were injected into the tail vein with  $2 \times 10^{10}$  particles of either AdSTK109 or AdhAATΔE1 diluted in phosphate-buffered saline (PBS). Mice were bled at the indicated time points and serum was frozen at  $-20^\circ\text{C}$ . An enzyme-linked immunosorbent assay (ELISA) was used to measure AAT levels in cell culture medium or in serum of mice, as previously described<sup>25</sup>.

Primer extension analyses. RNA was isolated from liver tissues 2 weeks after tail vein injection with  $2 \times 10^{10}$  particles of either vector. Primer

extension analyses were performed using liver RNA (20 µg) from injected and control animals, or RNA from human HepG2 cells (10 µg). The nucleotide sequence of the primer corresponded to nt. 101–124 relative to the start codon of the *PI* cDNA<sup>19</sup>.

Western-blot analyses. Different amounts of serum from control mice and transduced mice were fractionated by 10% SDS-PAGE, transferred to nitrocellulose filters and incubated with goat IgG fraction against human AAT (Cappel) as primary antibody and horseradish peroxidase conjugated rabbit anti-goat IgG (Pierce) as secondary antibody. Specific signals were detected by the ECL-method (Amersham).

Morphological analyses. Three transduced animals per time point were sacrificed at times as indicated and the livers were fixed in 10% formalin. Paraffin sections were stained with hematoxylin and eosin and examined in a blinded fashion. For immunostaining, cross sections were stained using a rabbit polyclonal antibody against Ki-67 as primary antibody (Novocastra) and a biotinylated goat anti-rabbit F(ab) fragment (Chemicon) as secondary antibody.

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## United States Patent [19]

[11] Patent Number: 5,801,154

Baracchini et al.

[45] Date of Patent: \*Sep. 1, 1998

[54] ANTISENSE OLIGONUCLEOTIDE  
MODULATION OF MULTIDRUG  
RESISTANCE-ASSOCIATED PROTEIN[75] Inventors: **Edgardo Baracchini**, San Diego; **C. Frank Bennett**, Carlsbad; **Nicholas M. Dean**, Encinitas, all of Calif.[73] Assignee: **Isis Pharmaceuticals, Inc.**, Carlsbad, Calif.

[\*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,510,239.

[21] Appl. No.: 835,770

[22] Filed: Apr. 8, 1997

## Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 628,731, Apr. 16, 1996, which is a continuation-in-part of Ser. No. 136,811, Oct. 18, 1993, Pat. No. 5,510,239.

[51] Int. Cl.<sup>6</sup> ..... C12N 15/85; A61K 48/00; C12Q 1/68; C07H 21/04

[52] U.S. Cl. .... 514/44; 435/6; 435/91.1; 435/171.3; 435/32.5; 536/23.1; 536/24.3; 536/24.5

[58] Field of Search ..... 435/6, 91.1, 320.1, 435/325, 366; 536/23.1, 24.5, 24.3; 514/44

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Primary Examiner—John L. LeGuyader  
Attorney, Agent, or Firm—Law Offices of Jane Massey Licata

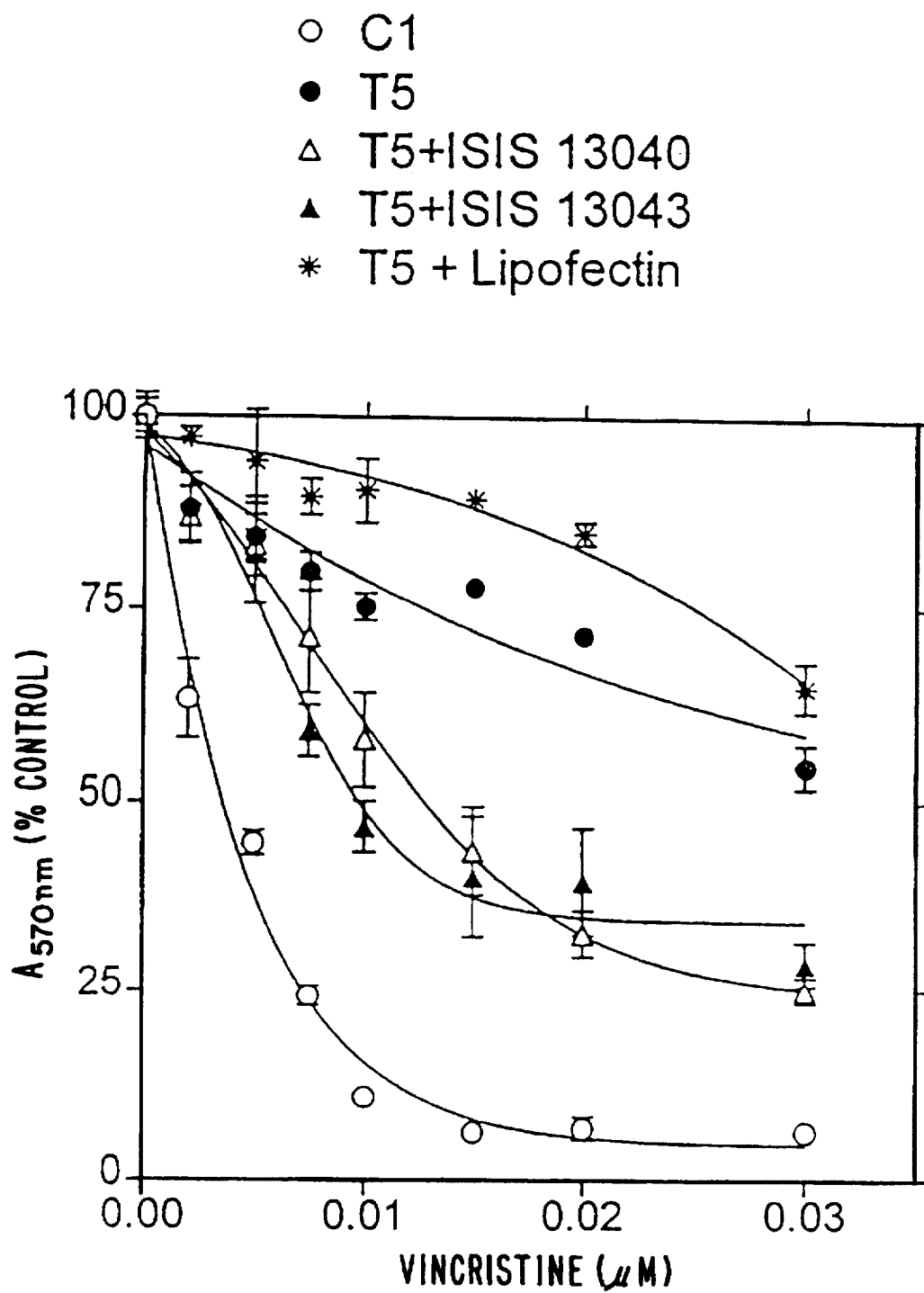
## [57] ABSTRACT

Compositions and methods are provided for the treatment and diagnosis of diseases or conditions amenable to treatment through modulation of the synthesis or metabolism of multidrug resistance-associated protein (MRP). In accordance with referred embodiments, oligonucleotides are provided which are specifically hybridizable with nucleic acids encoding MRP. In a preferred embodiment, the oligonucleotide has at least one 2'-methoxyethoxy modification. Methods of preventing the development of multidrug resistance and of improving the efficacy of chemotherapy are also disclosed.

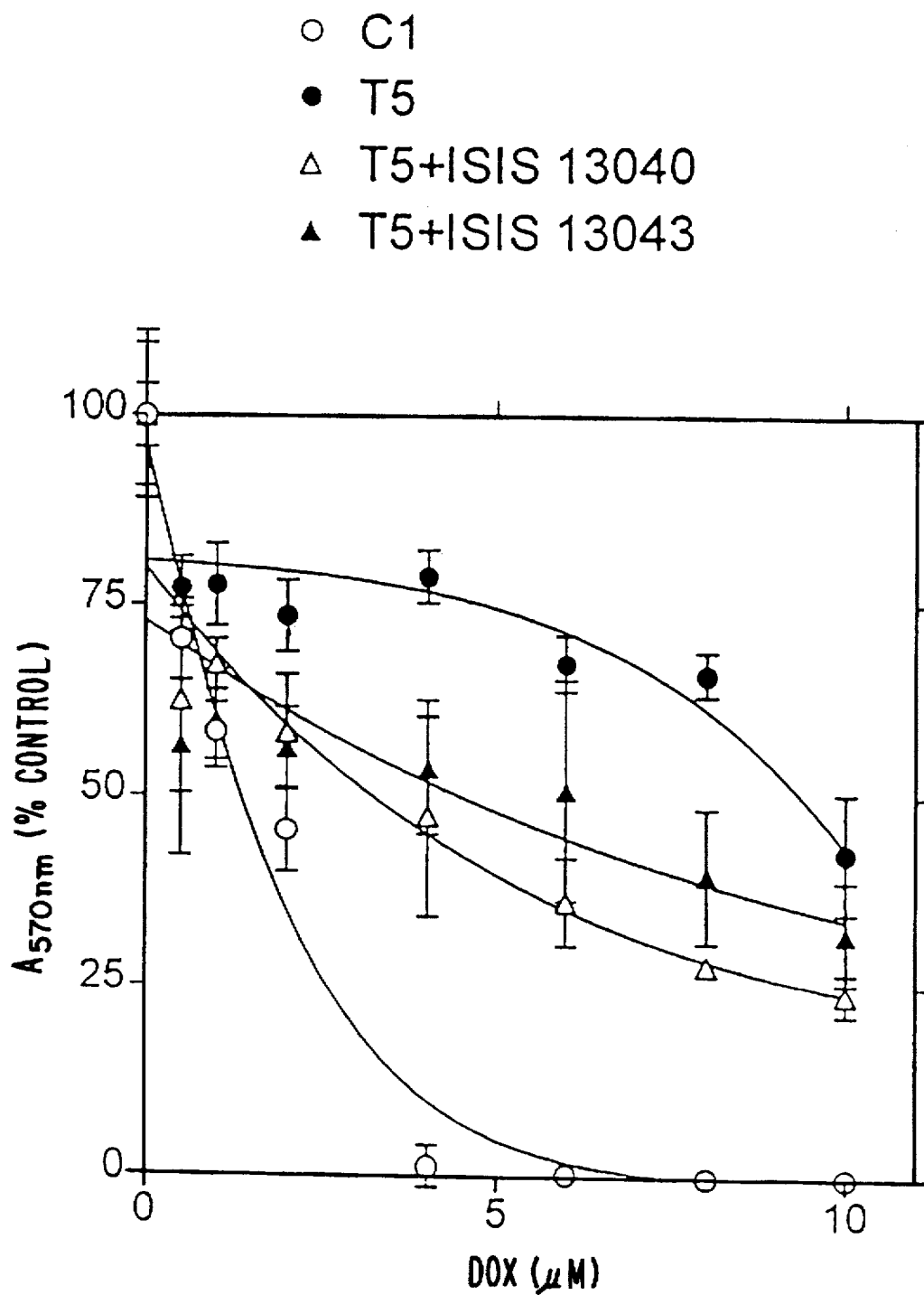
35 Claims, 11 Drawing Sheets

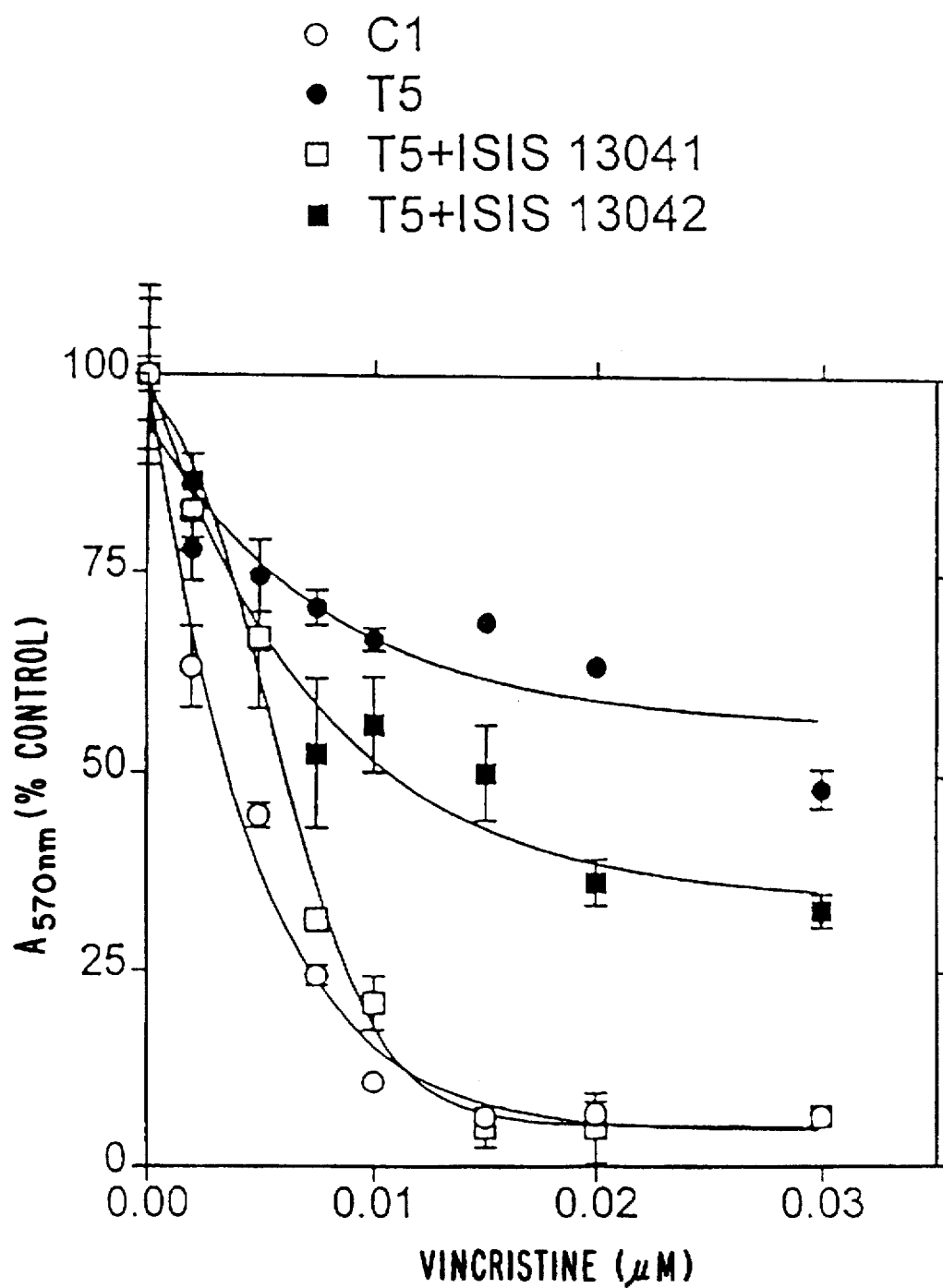
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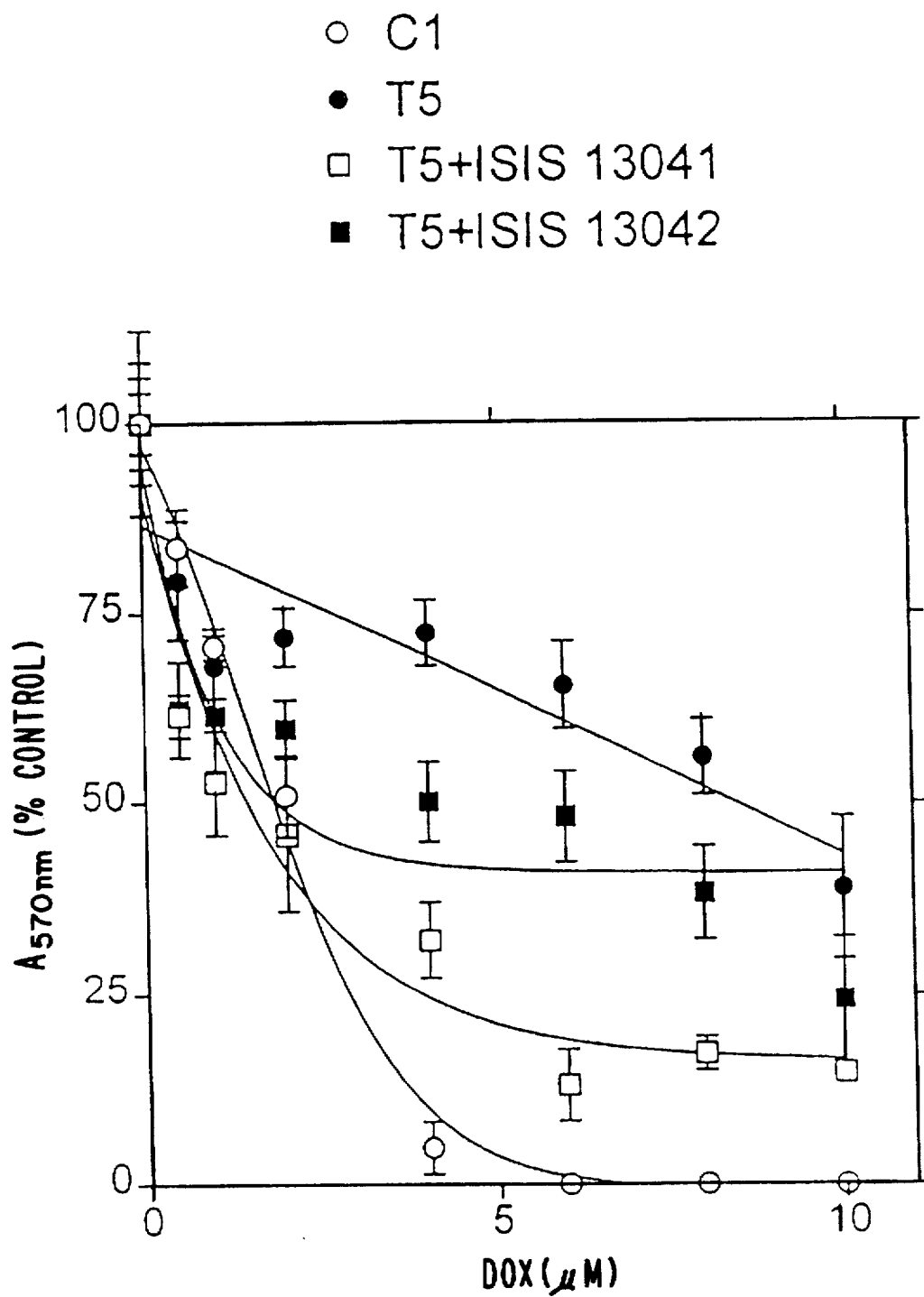


***Fig. 1A***

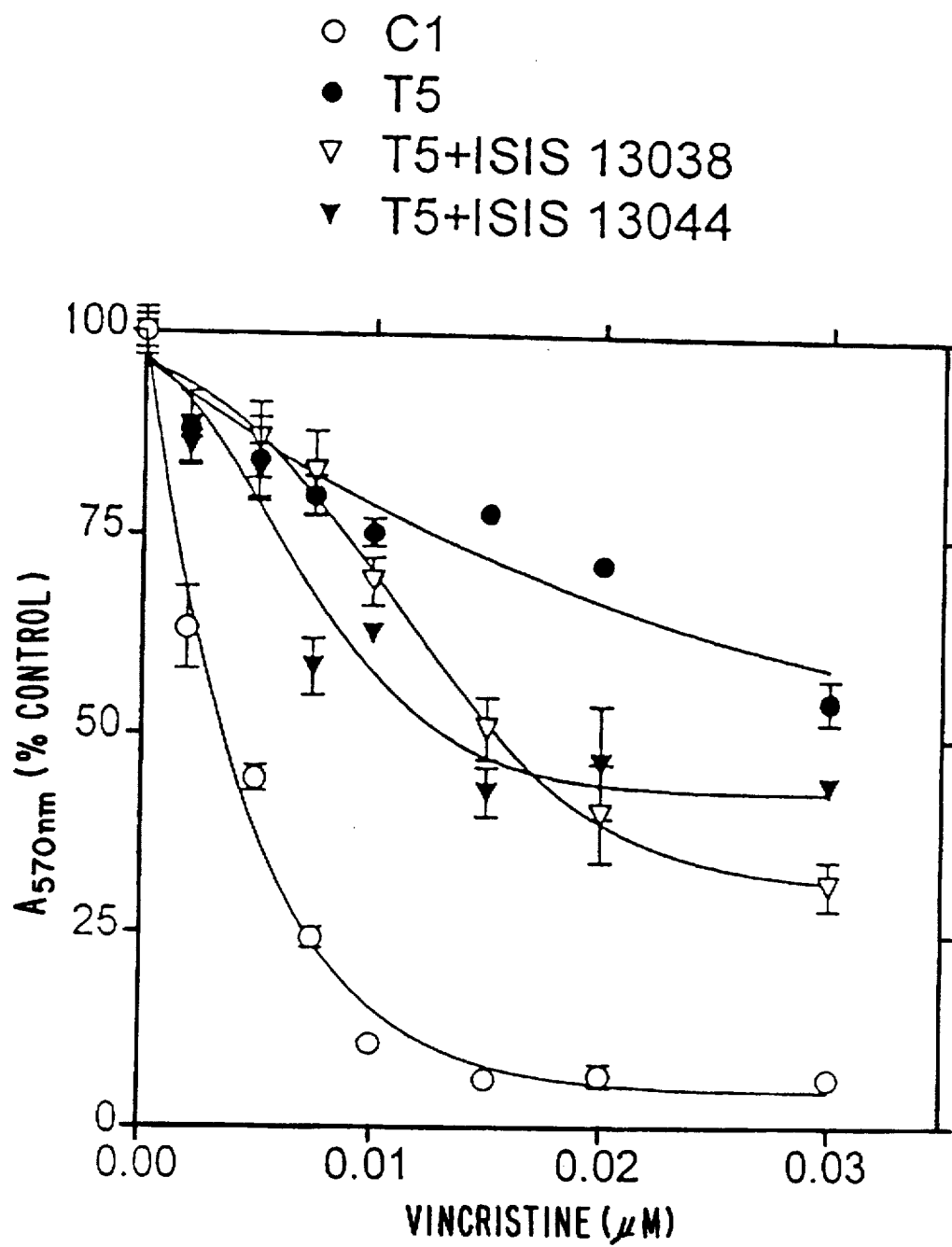
***Fig. 1B***



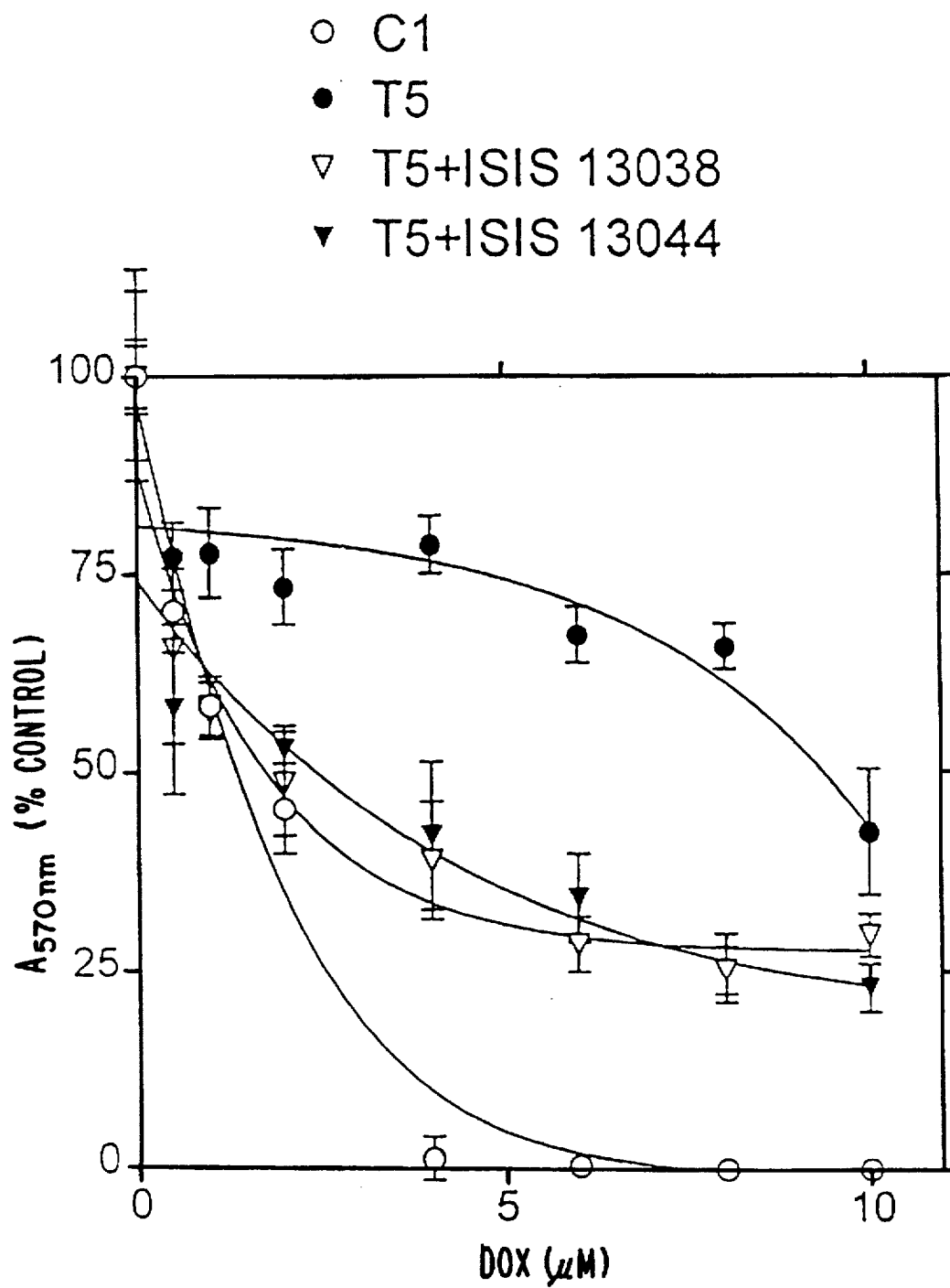
***Fig. 2A***

***Fig. 2B***

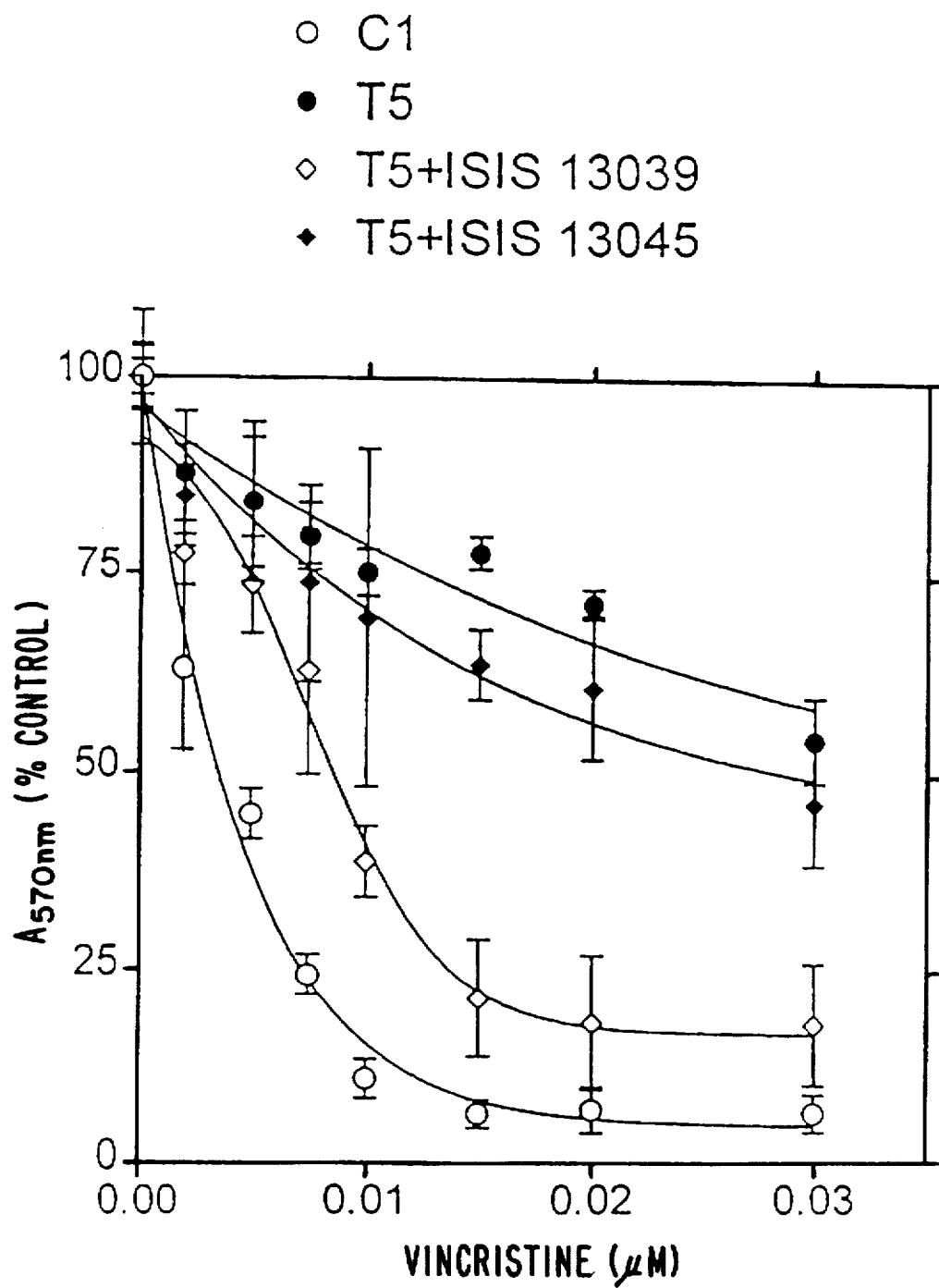


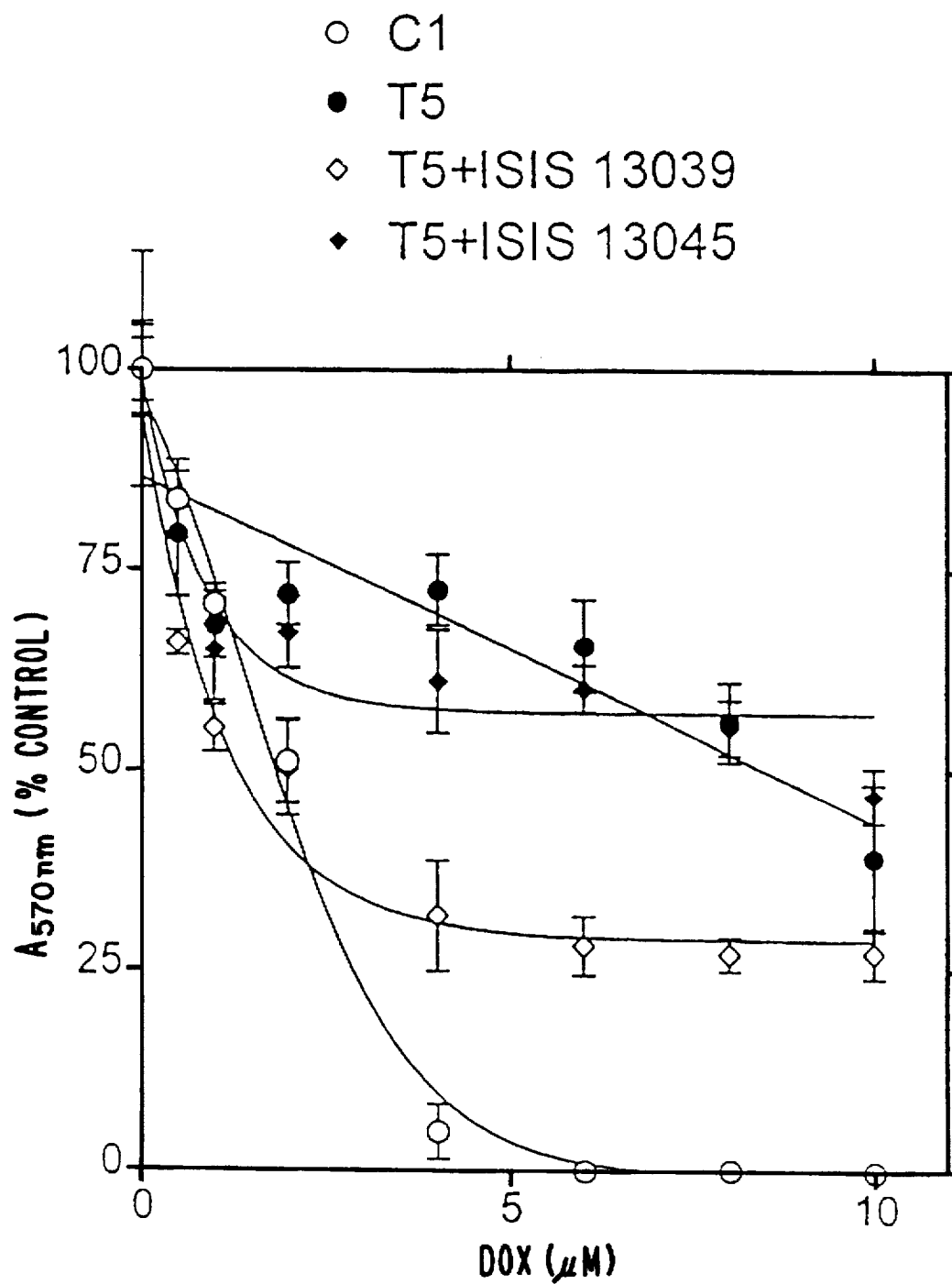


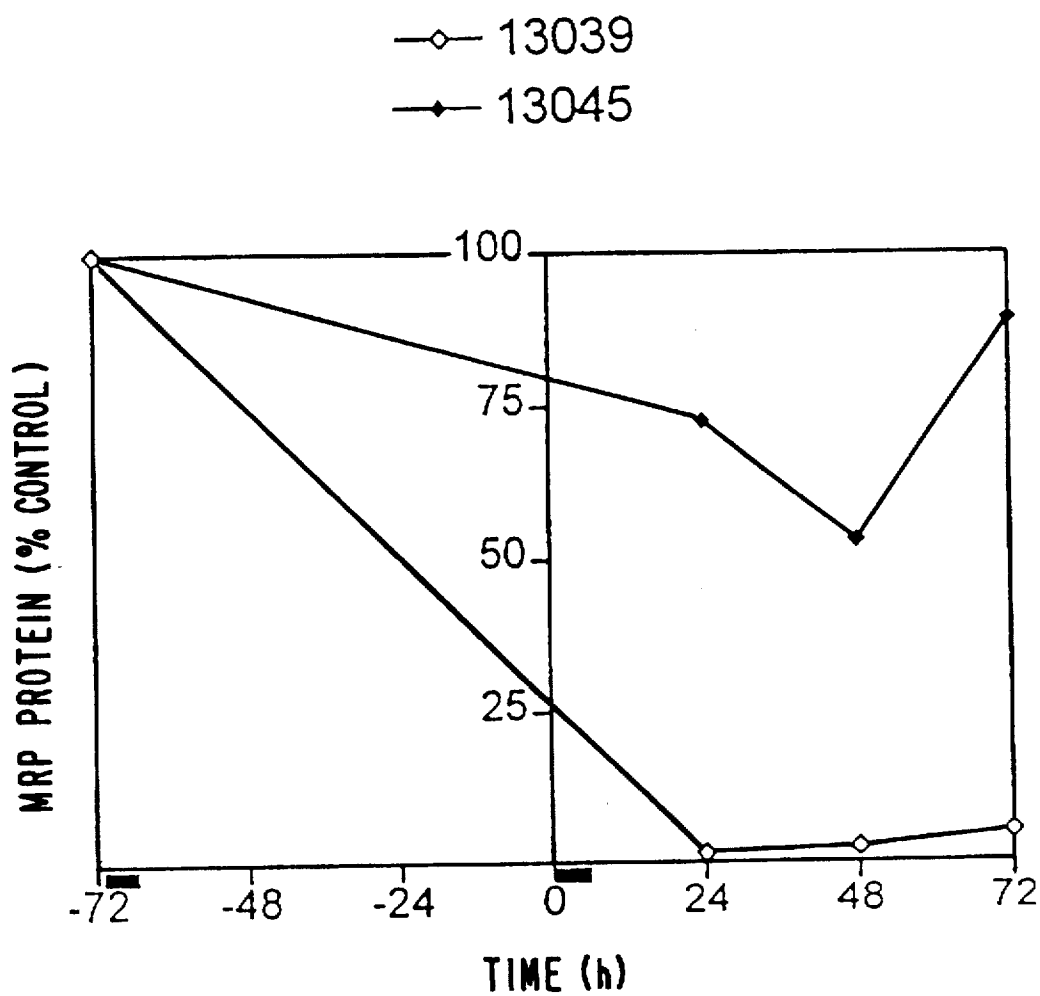
***Fig. 3A***



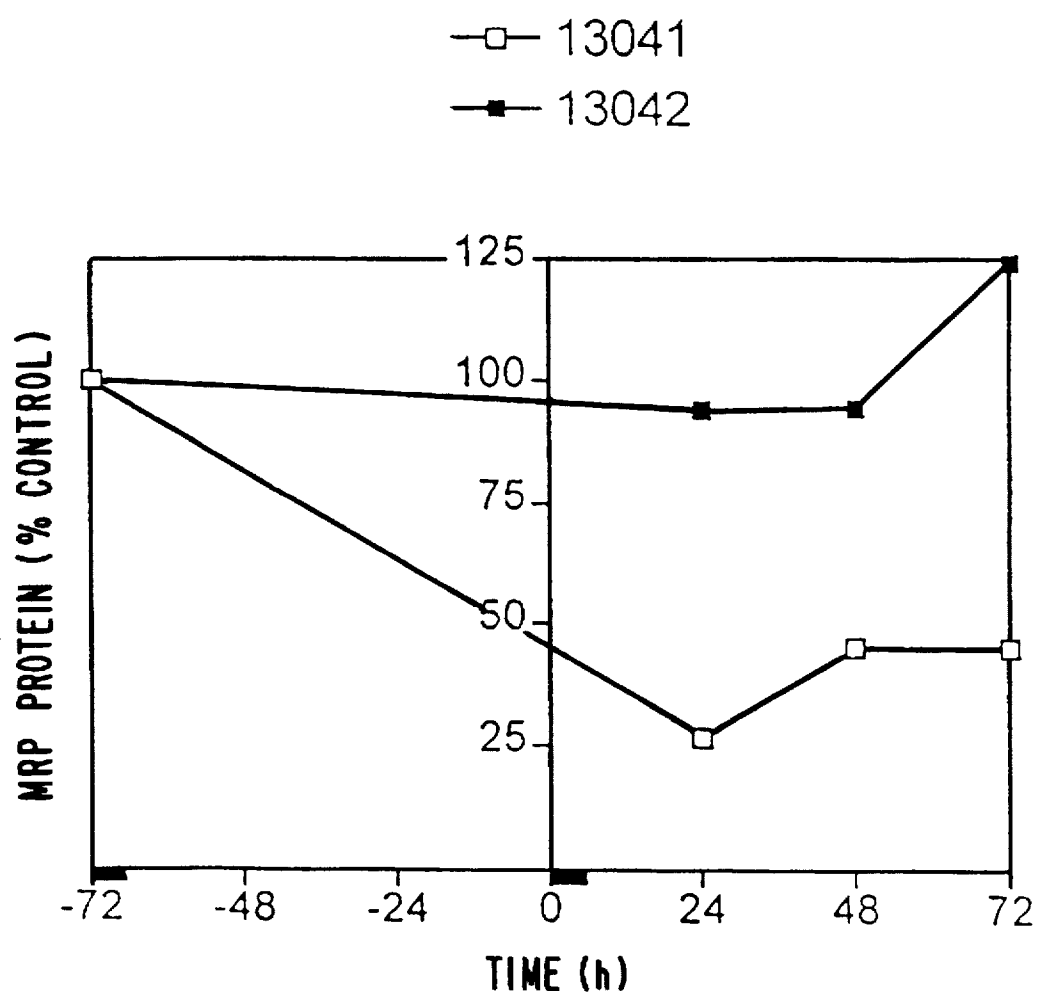
***Fig. 3B***

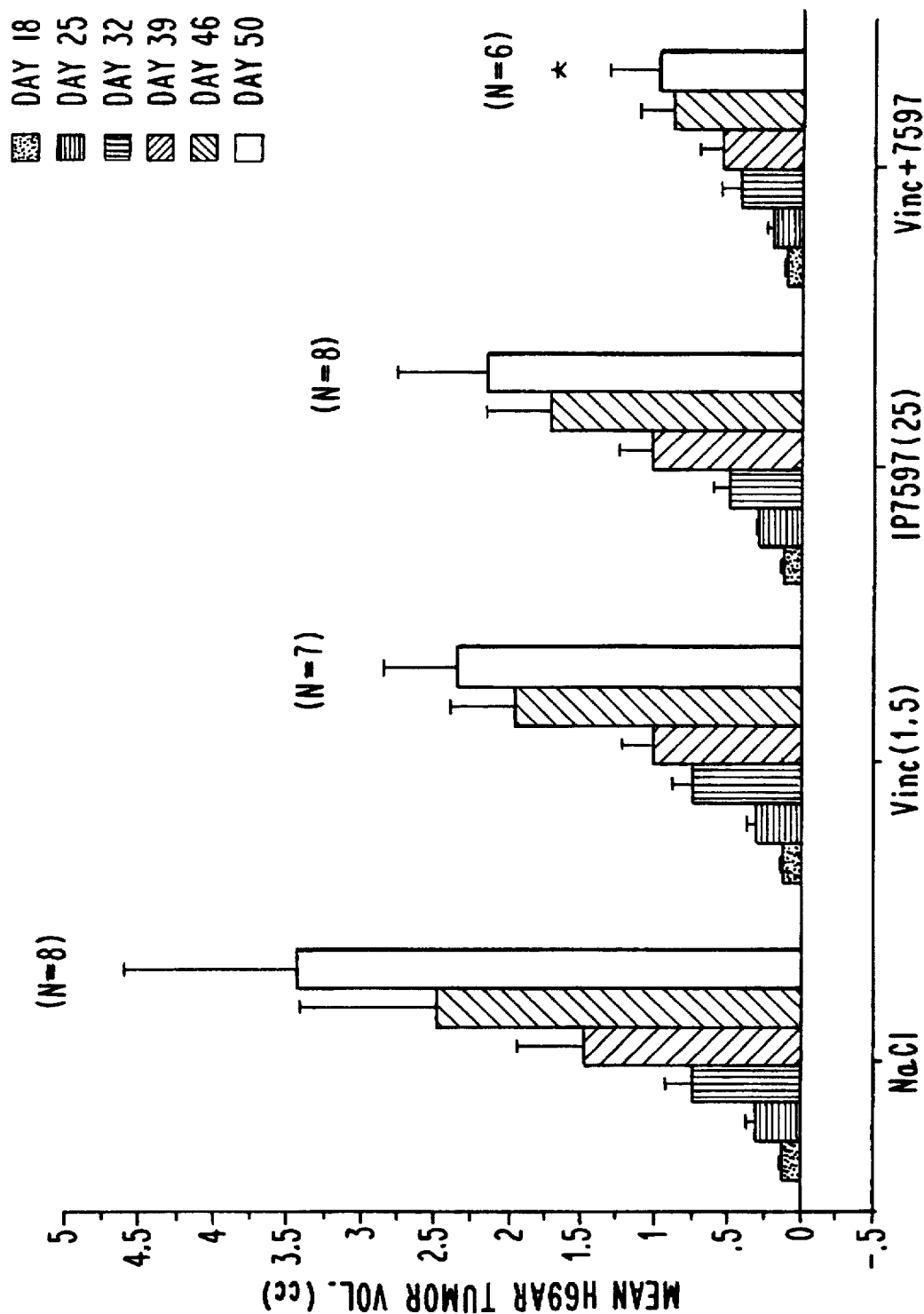
***Fig. 4A***

***Fig. 4B***



***Fig. 5A***

***Fig. 5B***



**Fig. 6**

# ANTISENSE OLIGONUCLEOTIDE MODULATION OF MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN

## RELATED APPLICATIONS

This application is a continuation-in-part of U.S. patent application Ser. No. 08/628,731, filed and U.S. patent application Ser. No. 08/136,811, filed Oct. 18, 1993, now issued as U.S. Pat. No. 5,510,239.

## FIELD OF THE INVENTION

This invention relates to diagnostics, research reagents and therapies for multidrug resistance and for disease states which respond to modulation of the phenomenon of multidrug resistance. In particular, this invention relates to antisense oligonucleotide interactions with certain messenger ribonucleic acids (mRNAs) or DNAs involved in the synthesis of the multidrug resistance-associated protein (MRP). Antisense oligonucleotides designed to hybridize to the mRNA encoding MRP are provided. These oligonucleotides have been found to lead to the modulation of the activity of the RNA or DNA, and thus to the modulation of the synthesis and metabolism of MRP. Palliation and therapeutic effect result. These oligonucleotides can also be used in assays and diagnostics, and can be useful in distinguishing MRP-associated multidrug resistance from other multidrug resistance pathways.

## BACKGROUND OF THE INVENTION

Acquired resistance to chemotherapy is a major problem in treatment of cancer by conventional cytotoxic drugs. Tumors may initially respond well to chemotherapy but later become resistant to a variety of unrelated drugs, leading to relapse. Multidrug resistance can arise via one of several independent pathways, any or all of which may be amenable to inhibition. One cause of multidrug resistance is believed to be overexpression of a transmembrane transport protein known as P-glycoprotein or MDR protein. Another distinct cause of multidrug resistance is believed to be overexpression of a member of the ATP-binding cassette transmembrane transporter superfamily known as multidrug resistance-associated protein (MRP). This protein is overexpressed in certain tumor cell lines which are multidrug resistant but do not overexpress P-glycoprotein. Cole et al. *Science* 1992, 258, 1650-1654; Slovak et al. *Cancer Res.* 1993, 53, 3221-3225. The gene encoding MRP was initially isolated from a multidrug-resistant small-cell lung cancer cell line. Small-cell lung cancer accounts for 20-25% of all lung cancer. Up to 90% of small-cell lung cancers respond initially to chemotherapy, but nearly all become multidrug resistant, leading to relapse.

MRP, unlike P-glycoprotein, has been shown to be a primary active ATP-dependent transporter of leukotrienes and other conjugated organic anions. Gao et al. *J. Biol. Chem.* 1996, 271, 27782-27787.

Compositions and methods for modulating and detecting MRP are the subject of this invention. Agents capable of reversing the phenomenon of multidrug resistance and thus "sensitizing" the drug resistant tumors to chemotherapy are desired. Cyclosporin A and other agents are able to reverse doxorubicin resistance in cells which overexpress MDR, but clinical use of these compounds is limited by their cytotoxicity. Further, these reversing agents do not work in cells which overexpress MRP. Antisense oligonucleotides targeted to the MDR mRNA encoding P-glycoprotein have

been used to inhibit the synthesis of P-glycoprotein (MDR protein) and to partially reverse the multidrug resistance phenotype. Thierry et al. *Biochem. Biophys. Res. Comm.* 1993, 190, 952-960; Vasanthakumar, G. and N. K. Ahmed *Cancer Commun.* 1989, 1, 225-232.

While compositions and methods for reversing P-glycoprotein (MDR)-associated multidrug resistance or MDR synthesis have shown limited success, these are not targeted to the same target as the compositions and methods of the present invention. Consequently there remains a long-felt need for compositions and methods for modulation and diagnosis of other types of multidrug resistance. Oligonucleotides that are specifically hybridizable with MRP mRNA are desired for their diagnostic and therapeutic utility. Interference with MRP expression is desired as a means of reversing the multidrug resistance phenomenon, and making a distinction between multidrug resistance due to MRP and that due to other causes. Interference with MRP expression is also desired for improving the efficacy of conventional methods of cancer chemotherapy, particularly of lung cancer, most particularly of small-cell lung cancer.

Compositions and methods for treating inflammatory conditions are also desired. The role of leukotrienes in inflammatory conditions, particularly asthma, inflammatory bowel disease, rheumatoid arthritis and psoriasis, is well known and leukotriene inhibitors and antagonists are being examined as drugs. Henderson, W. R., Jr., *Ann. Int. Med.* 1994, 121, 684-697. Leukotriene release has also been implicated in other inflammatory conditions including allergic rhinitis, cystic fibrosis, adult respiratory distress syndrome, and glomerulonephritis. Because MRP functions as a primary ATP-dependent transporter of cysteinyl leukotrienes, compositions and methods for modulating MRP expression are believed to have therapeutic utility for these and other inflammatory conditions in which leukotriene release is involved.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1a and 1b are line drawings showing the reversal of resistance of HeLa T5 cells to vincristine (FIG. 1a) or doxorubicin (FIG. 1b) (escalating doses along the abscissa) after treatment with ISIS 13040 or its scrambled control, ISIS 13043. Open circles=C1 control cells; solid circles=untreated T5 cells; open triangles=T5 cells+ISIS 13040; closed triangles=T5 cells+ISIS 13043; asterisks=LIPOFECTIN™ alone.

FIGS. 2a and 2b are line drawings showing the reversal of resistance of HeLa T5 cells to vincristine (FIG. 2a) or doxorubicin (FIG. 2b) (escalating doses along the abscissa) after treatment with ISIS 13041 or its scrambled control, ISIS 13042. Open circles=C1 control cells; solid circles=untreated T5 cells; open squares=T5 cells+ISIS 13041; closed squares=T5 cells+ISIS 13042.

FIGS. 3a and 3b are line drawings showing the reversal of resistance of HeLa T5 cells to vincristine (FIG. 3a) or doxorubicin (FIG. 3b) (escalating doses along the abscissa) after treatment with ISIS 13038 or its scrambled control, ISIS 13044. Open circles=C1 control cells; solid circles=untreated T5 cells; open inverted triangles=T5 cells+ISIS 13038; closed inverted triangles=T5 cells+ISIS 13044.

FIGS. 4a and 4b are line drawings showing the reversal of resistance of HeLa T5 cells to vincristine (FIG. 4a) or doxorubicin (FIG. 4b) (escalating doses along the abscissa) after treatment with ISIS 13039 or its scrambled control, ISIS 13045. Open circles=C1 control cells; solid circles=untreated T5 cells; open diamonds=T5 cells+ISIS 13039; closed diamonds=T5 cells+ISIS 13045.



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FIG. 5 is a line graph showing the suppression of MRP protein levels after treatment with antisense oligonucleotides. FIG. 5a shows the results of treatment with ISIS 13039 (open diamonds) or its scrambled control, ISIS 13045 (closed diamonds). FIG. 5b shows the results of treatment with ISIS 13041 (open squares) or its scrambled control, ISIS 13042 (closed squares).

FIG. 6 is a bar graph showing inhibition of H69AR tumor growth in nude mice at six time points after treatment with ISIS 7597 (IP7597), vincristine (Vinc), ISIS 7597 plus vincristine (Vinc+7597) or saline control (NaCl).

### SUMMARY OF THE INVENTION

In accordance with the present invention, oligonucleotides are provided which specifically hybridize with nucleic acids encoding multidrug resistance-associated protein (MRP). The oligonucleotides are designed to bind either directly to mRNA or to a selected DNA portion forming a triple stranded structure, thereby modulating the amount of mRNA made from the gene. In either case, expression of MRP protein is ultimately modulated. "Hybridization," in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which are known to form two hydrogen bonds between them. "Specifically hybridizable" indicates a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences. It is well known in the art that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable.

The relationship between an oligonucleotide and its complementary target nucleic acid is commonly denoted as "antisense". In the context of the present invention, the "target" is a nucleic acid encoding multi-drug resistance-associated protein (MRP); in other words, the MRP gene or mRNA expressed from the MRP gene.

It is preferred to target specific genes for antisense attack. It has been discovered that the gene coding for MRP is particularly useful for this approach. Inhibition of MRP expression is expected to be useful for the treatment of multidrug resistance. However, "modulation" in the context of this invention means either an increase or decrease (stimulation or inhibition) of MRP expression.

Methods of modulating the synthesis of MRP in cells and tissues comprising contacting an animal suspected of having multidrug-resistant cells or tissues with an oligonucleotide specifically hybridizable with nucleic acids encoding the MRP protein are provided.

Methods of treating an animal suspected of having a condition characterized by elevated levels of MRP are also provided. Such methods comprise administering to an animal a therapeutically effective amount of an oligonucleotide specifically hybridizable with nucleic acids encoding the MRP protein.

Other aspects of the invention are directed to methods for improving the efficacy of chemotherapy and preventing the development of multidrug resistance during chemotherapeutic drug treatment of a disease. Such methods comprise administering to an animal an appropriate amount of oligonucleotide specifically hybridizable with nucleic acids encoding the MRP protein in conjunction with a chemotherapeutic drug treatment.

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Methods for diagnosis are also a part of this invention, and include methods for determining MRP-associated multidrug resistance as being distinct from other pathways of multidrug resistance development. Such methods comprise contacting cells or tissues or bodily fluids from the diseased animals with oligonucleotides in accordance with this invention in order to detect MRP overexpression.

### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Oligonucleotides have recently become accepted as drugs for the treatment of disease states in animals and man. For example, workers in the field have now identified antisense, triplex and other oligonucleotide therapeutic compositions which are capable of modulating expression of genes implicated in viral, fungal and metabolic diseases. Numerous antisense oligonucleotide drugs have been safely administered to humans and a number of clinical trials are presently underway. Efficacy has been demonstrated for several oligonucleotide drugs, directed to both viral and cellular gene targets. It is thus established that oligonucleotides can be useful therapeutics.

For therapeutics, an animal suspected of having a disease which can be treated by decreasing the expression of MRP is treated by administering oligonucleotides in accordance with this invention. Oligonucleotides may be formulated in a pharmaceutical composition, which may include carriers, thickeners, diluents, buffers, preservatives, surface active agents, liposomes or lipid formulations and the like in addition to the oligonucleotide. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like in addition to oligonucleotide. Oligonucleotides may be administered in conjunction with conventional cancer chemotherapeutic drugs which are well known to those skilled in the art.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration. Oligonucleotides with at least one 2'-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may also be included.

Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The formulation of therapeutic compositions and their subsequent administration is within the skill of those in the art. Dosing is dependent on severity and responsiveness of

the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on  $EC_{50}$ s found to be effective in vitro and in vivo animal models. In general, dosage is from 0.01  $\mu$ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily determine repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01  $\mu$ g to 100 g per kg of body weight, once or more daily, to once every 20 years.

The present invention is also suitable for detection of MRP overexpression in tissue or other samples from patients who have developed multidrug resistance. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection, and usually quantitation, of such inhibition. For example, radiolabeled oligonucleotides can be prepared by  $^{32}$ P labeling at the 5' end with polynucleotide kinase. Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, Volume 2, pg. 10.59. Radiolabeled oligonucleotides are then contacted with tissue or cell samples suspected of MRP overexpression or with RNA extracted from such samples. The sample is then washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide (which in turn indicates expression of the nucleic acids encoding MRP) and can be quantitated using a scintillation counter or other routine means. Comparison to appropriate controls allows overexpression of MRP to be determined. Radiolabeled oligonucleotide can also be used to perform autoradiography of tissues to determine the localization, distribution and quantitation of MRP overexpression for research, diagnostic and therapeutic purposes. In such studies, tissue sections are treated with radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to routine autoradiography procedures. The emulsion, when developed, yields an image of silver grains over the regions expressing MRP. Quantitation of the silver grains permits MRP overexpression to be detected.

Analogous assays for fluorescent detection of MRP expression can be developed using oligonucleotides of the invention which are conjugated with fluorescein or other fluorescent tag instead of radiolabeling. Such conjugations are routinely accomplished during solid phase synthesis using fluorescently-labeled amidites or CPG (e.g., fluorescein-labeled amidites or CPG available from Glen Research, Sterling, Va. See 1993 Catalog of Products for DNA Research, Glen Research, Sterling, Va., pg. 21).

Each of these assay formats is known in the art. One of skill could easily adapt these known assays for detection of MRP expression in accordance with the teachings of the invention providing a novel and useful means to detect MRP expression.

In addition, the ability of the oligonucleotides of the present invention to inhibit MRP synthesis in cultured diseased cells is extremely useful in distinguishing drug resistance which is MRP-associated from that which arises via another pathway. In case of a disease state such as cancer, oligonucleotide-treated cells from the drug resistant tumor sites can be cultured and screened for reversal of drug resistance, i.e., increased sensitivity to chemotherapeutic drugs as quantitated by a decrease in the  $IC_{50}$  values. This information can be used to treat the disease state more efficaciously.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which would be appreciated by persons of ordinary skill in the art.

The present invention employs oligonucleotides for use in antisense inhibition of the function of RNA and DNA encoding multidrug resistance-associated protein (MRP). In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. A discussion of antisense oligonucleotides and some desirable modifications can be found in De Mesmaeker et al., *Acc. Chem. Res.* 1995, 28, 366-374.

Specific examples of some preferred oligonucleotides envisioned for this invention include those containing modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly  $CH_2-NH-O-CH_2$ ,  $CH_2-N(CH_3)-O-CH_2$  [known as a methylene (methylimino) or MMI backbone],  $CH_2-O-N(CH_3)-CH_2$ ,  $CH_2-N(CH_3)-N(CH_3)-CH_2$  and  $O-N(CH_3)-CH_2-CH_2$  backbones, wherein the native phosphodiester backbone is represented as  $O-P-O-CH_2$ . The amide backbones disclosed by De Mesmaeker et al. (*Acc. Chem. Res.* 1995, 28, 366-374) are also preferred. Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Pat. No. 5,034,506). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the nitrogen atoms of the polyamide backbone (Nielsen et al., *Science* 1991, 254, 1497).

Oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH, SH,  $SCH_3$ , F, OCN,  $OCH_3OCH_3$ ,  $OCH_3O(CH_2)_nCH_3$ ,  $O(CH_2)_nNH_2$  or  $O(CH_2)_nCH_3$  where n is from 1 to about 10;  $C_1$  to  $C_{10}$  lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN;  $CF_3$ ;  $OCF_3$ ; O-, S-, or N-alkyl; O-, S-, or N-alkenyl;  $SOCH_3$ ;  $SO_2CH_3$ ;  $ONO_2$ ;  $NO_2$ ;  $N_3$ ;  $NH_2$ ; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a

reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl)] (Martin et al., *Helv. Chim. Acta* 1995, 78, 486). Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-propoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Oligonucleotides may also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2'deoxyctosine and often referred to in the art as 5-me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N<sup>6</sup>(6-aminohexyl)adenine and 2,6-diaminopurine. Kornberg, A., *DNA Replication*, W. H. Freeman & Co., San Francisco, 1980, pp 75-77; Gebeyehu, G., et al. *Nucl. Acids Res.* 1987, 15,4513). A "universal" base known in the art, e.g., inosine, may be included. 5-me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6°-1.2° C. (Sanghvi, Y. S., in *Antisense Research and Applications*, Crooke and Lebleu, eds., CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger et al. *Proc. Natl. Acad. Sci. USA* 1989, 86, 6553), cholic acid (Manoharan et al. *Bioorg. Med. Chem. Lett.* 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al. *Ann. N.Y. Acad. Sci.* 1992, 660, 306; Manoharan et al. *Bioorg. Med. Chem. Lett.* 1993, 3, 2765), a thiocholesterol (Oberhauser et al. *Nucl. Acids Res.* 1992, 20, 533), an aliphatic chain, e.g., dodecanediol or undecyl residues (Saison-Behmoaras et al. *EMBO J.* 1991, 10, 111; Kabanov et al. *FEBS Lett.* 1990, 259, 327; Svinarchuk et al. *Biochimie* 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651; Shea et al. *Nucl. Acids Res.* 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al. *Nucleosides & Nucleotides* 1995, 14, 969), or adamantane acetic acid (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651), a palmitoyl moiety (Mishra et al. *Biochim. Biophys. Acta* 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al. *J. Pharmacol. Exp. Ther.* 1996, 277, 923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S. Pat. Nos. 5,138,045, 5,218,105 and 5,459,255.

The oligonucleotides of the invention may be provided as prodrugs, which comprise one or more moieties which are cleaved off, generally in the body, to yield an active oligonucleotide. One example of a prodrug approach is described by Imbach et al. in WO Publication 94/26764.

It is not necessary for all positions in a given oligonucleotide to be uniformly modified and, in fact, more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even within a single nucleoside within an oligonucleotide. The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligos are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. Typically, chimeric oligonucleotides are "gapped" oligonucleotides (or "gapmers") in which a region of deoxynucleotides (the "gap"), preferably containing at least four contiguous deoxynucleotides, is flanked by regions of modified nucleotides, preferably 2'-sugar modified nucleotides. In a preferred embodiment, the flanking regions (or "wings") contain 2'-alkoxy or 2'-alkoxyalkoxy modifications, more preferably 2'-methoxyethoxy. In preferred embodiments the backbone may be phosphorothioate throughout or may be phosphodiester in the "wings" and phosphorothioate in the "gap". In other preferred embodiments, chimeric oligonucleotides may be "winged" oligonucleotides (or "wingmers" or hemichimeras) in which there is a deoxy "gap", preferably at least 4 contiguous deoxynucleotides, flanked on either the 5' or the 3' side by a region of modified nucleotides. Again, the flanking region (or "wing") preferably contains 2'-alkoxy or 2'-alkoxyalkoxy modifications, more preferably 2'-methoxyethoxy and the backbone may be phosphorothioate throughout or may be phosphodiester in the "wing" and phosphorothioate in the "gap". Other configurations of chimeric oligonucleotide are also comprehended by this invention. These may involve other modifications of the sugar, base or backbone, preferably in the oligonucleotide wing(s).

The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 30 nucleotides. It is more preferred that such oligonucleotides comprise from about 12 to 25 nucleotides. As will be appreciated, a nucleotide is a base-sugar combination suitably bound to an adjacent nucleotide through phosphodiester or other bonds.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis

may also be employed; however, the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

It is preferred to target specific genes for antisense attack. "Targeting" an oligonucleotide to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding multidrug resistance-associated protein. The targeting process also includes determination of a site or sites within this gene for the oligonucleotide interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Because, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding MRP, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. The open reading frame (ORF) or "coding region", which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene) and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termi-

nation codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene). The transcription initiation site, or "5' cap site" and the 5' cap region (which encompasses from about 25 to about 50 contiguous nucleotides at the extreme 5' terminus of a capped mRNA) may also be effective targets. mRNA splice sites may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Where gene deletion rearrangements exist, aberrant fusion junctions are also preferred targets. Once the target site has been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect under conditions in which specific hybridization is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment or, in the case of in vitro methods, under conditions in which the methods are used. In preferred embodiments of the present invention, the oligonucleotides are specifically hybridizable with a transcription initiation site, a translation initiation site, coding sequences and sequences in the 5'- and 3'-untranslated regions of mRNA encoding MRP.

MRP belongs to the superfamily of ATP-binding cassette transport systems. This family includes the cystic fibrosis transmembrane conductance regulator, P-glycoprotein, and other transport proteins. The human MRP protein is 1531 amino acids in length and is encoded by an mRNA which is approximately 6.5 kb in length. Cole et al. *Science* 1992, 258, 1650-1654; Cole et al. *Science* 1993, 260, 879 (sequence correction); Slovak et al. *Cancer Res.* 1993, 53, 3221-3225. Antisense oligonucleotides (shown in Table 1) were designed to be specifically hybridizable with sequences in the 5'-untranslated region, 3'-untranslated region and coding region of the MRP gene. The sequence of the MRP gene is available in publications [Cole et al. *Science* 1992, 258, 1650-1654; Cole et al. *Science* 1993, 260, 879 (sequence correction)] or through Genbank accession number L05628.

TABLE 1

Antisense Oligonucleotides Specifically Hybridizable With MRP  
(All are phosphorothioates; ISIS 7607 is also 2'-O-methyl)

ISIS #	TARGET REGION	SEQUENCE	SEQ ID NO:
7607	5' UTR	CGG GGC CGC AAC GCC GCC UG	1
7608	5' UTR	CGG GGC CGC AAC GCC GCC TG	2
7606	5' UTR	GGT GAT CGG GCC CGG TTG CT	3
7595	5' UTR	CCG GTG GCG CGG GCG GCG GC	4
7592	AUG	AGC CCC GGA GCG CCA TGC CG	5
7593	Coding	TCG GAG CCA TCG GCG CTG CA	6
7594	Coding	GGC ACC CAC ACG AGG ACC GT	7
7597	Coding	TGC TGT TCG TGC CCC CGC CG	8
7598	Coding	CGC GCT GCT TCT GGC CCC CA	9
7599	Coding	GCG GCG ATG GGC GTG GCC AG	10
7600	Coding	CAG GAG GTC CGA TGG GGC GC	11
7601	Coding	GCT CAC ACC AAG CCG GCG TC	12
7603	3' UTR	AGG CCC TGC AGT TCT GAC CA	13
7605	3' UTR	CTC CTC CCT GGG CGC TGG CA	14
7602	3' UTR	ACC GGA TGG CGG TGG CTG CT	15
7604	3' UTR	CGC ATC TCT GTC TCT CCT GG	16

Preferred oligonucleotides useful in the invention comprise one of these sequences, or part thereof.

H69AR cells were treated with phosphorothioate oligonucleotides (SEQ ID NO: 1-16) in the presence of LIPO-

FECTION™ (GIBCO/BRL) as described in the following examples. Oligonucleotides ISIS 7597 and ISIS 7598 (SEQ ID NO: 8 and SEQ ID NO: 9), both specifically hybridizable to the coding region of MRP, consistently inhibited steady-state MRP protein levels by greater than 30% compared to LIPOFECTION™ controls in multiple ELISA experiments. In one experiment, ISIS 7597 inhibited MRP protein levels by over 95%. Oligonucleotides ISIS 7597 and 7598 are therefore preferred. It should be noted that the ELISA assay measures steady-state levels of MRP protein; because of the long half-life of the MRP protein, complete inhibition of MRP protein synthesis would be expected to be reflected as a decrease, but not complete loss, of MRP protein in these assays. This level of inhibition in this assay is considered to be significant. In Northern blot analysis of the effects of ISIS 7597 and 7598 on MRP mRNA levels, both oligonucleotides were demonstrated to virtually eliminate MRP mRNA expression.

Based on results obtained with the oligonucleotides of Table 1, additional phosphorothioate oligonucleotides were designed. These oligonucleotides are shown in Table 2.

TABLE 2

Phosphorothioate Antisense Oligonucleotides Specifically Hybridizable With MRP			
ISIS #	TARGET REGION	SEQUENCE	SEQ ID NO:
8356	AUG	CAG AAG CCC CGG AGC GCC AT	17
8358	Coding	GCC CCC GCC GTC TTT GAC AG	18
8359	Coding	GTG ATG CTG TTC GTG CCC CC	19
8357	Coding	CTC ACG GTG ATG CTG TTC GT	20

TABLE 2-continued

Phosphorothioate Antisense Oligonucleotides Specifically Hybridizable With MRP			
ISIS #	TARGET REGION	SEQUENCE	SEQ ID NO:
8362	Coding	CCC CCA GAC AGG TTC ACG CC	21
8361	Coding	CTG GCC CCC AGA CAG GTT CA	22
8360	Coding	GCC AGG CTC ACG CGC TGC TT	23
8363	3' UTR	CAC AGC CAG TTC CAG GCA GG	24
8364	3' UTR	CCT GGG TCT TCA CAG CCA GT	25

Chimeric oligonucleotides having SEQ ID NO: 8 were prepared. These oligonucleotides had uniform phosphorothioate backbones and central "gap" regions of 8 deoxynucleotides flanked by 2 regions of 2'-O-propyl modified nucleotides (ISIS 9659) or 2'-fluoro modified nucleotides (ISIS 9661).

Further, chimeric oligonucleotides having SEQ ID NO: 9 were prepared. These oligonucleotides had uniform phosphorothioate backbones and central "gap" regions of 8 deoxynucleotides flanked by 2 regions of 2'-O-propyl modified nucleotides (ISIS 9660) or 2'-fluoro modified nucleotides (ISIS 9662).

Additional oligonucleotides targeted to human MRP were synthesized. These are shown in Table 3. ISIS 9659, 9661, 11471, 11468 and 11469 have all been shown to inhibit MRP expression in a dose- and sequence-dependent manner. Canitrot et al. *Anti-Cancer Drugs* 1996, 7(suppl. 3), 93-99.

TABLE 3

Antisense Oligonucleotides Targeted to MRP				
ISIS #	TARGET REGION	SEQUENCE	MODIFICATION	SEQ ID NO:
9567	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-F	8
11468	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-F	8
11469	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-F	8
11584	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-F	8
11585	Coding	CGC GCT GCT TCT GGC CCC CA	PS/2'-F	9
11586	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-F	8
11471	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-O-propyl	8
11077	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-O-hexylamino-cholesterol	8
9658	Sense	CGG CGG GGG CAC GAA CAG CA	PS	26
12680	Sense	CGG CGG GGG CAC GAA CAG CA	PS/2'-O-propyl	26

TABLE 4

Antisense Oligonucleotides Targeted to MRP				
ISIS #	TARGET REGION	SEQUENCE	MODIFICATION	SEQ ID NO:
13038	Coding	TGC TGT TCG TGC CCC CGC CG	PO/PS/2'-MOE	8
13039	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-MOE	8
13040	Coding	TGC TGT TCG TGC CCC CGC CG	PO/PS/2'-MOE	8
13041	Coding	TGC TGT TCG TGC CCC CGC CG	PS/2'-MOE	8
15479	Sense control	CGG CGG GGG CAC GAA CAG CA	PS/2'-MOE	26
15480	Sense control	CGG CGG GGG CAC GAA CAG CA	PS/2'-MOE	26
13042	Scrambled	TCG TGG CCG CGT TCT CCC CG	PS/2'-MOE	27
13043	Scrambled	TCG TGG CCG CGT TCT CCC CG	PO/PS/2'-MOE	27
13044	Scrambled	TCG TGG CCG CGT TCT CCC CG	PO/PS/2'-MOE	27
13045	Scrambled	TCG TGG CCG CGT TCT CCC CG	PS/2'-MOE	27
15477	Scrambled	CGT GTT GCT CGT GCC CGC CC	PS/2'-MOE	28
15478	Scrambled	CGT GTT GCT CGT GCC CGC CC	PS/2'-MOE	28

Reversal of drug resistance in HeLa T5 cells following treatment with 2-methoxyethoxy antisense oligonucleotides

T5 cells are drug-resistant transfected HeLa cells which express elevated levels of MRP. They were obtained by stable transfection with an MRP cDNA expression vector. C1 HeLa cells were transfected with the vector alone and serve as controls. Canitrot et al. *Anti-Cancer Drugs* 1996, 7 (suppl. 3), 93-99. T5 and C1 cells were treated with antisense oligonucleotide (500 nM in the presence of LIPOFECTINTM) as described in Canitrot et al., supra, except that cells were treated twice with oligonucleotide, on days 1 and 4 after plating, and then exposed to either vincristine or doxorubicin on day 5. The MTT assay for chemosensitivity was done on day 8.

The oligonucleotides tested were chimeric 2'-methoxyethoxy oligonucleotides: ISIS 13038 (mixed PS/PO backbone) and ISIS 13039 (PS backbone), each of which has a 12-deoxynucleotide gap; their scrambled controls, ISIS 13044 and 13045, respectively; ISIS 13040 (mixed PS/PO backbone) and ISIS 13041 (PS backbone), each of which has a 8-deoxynucleotide gap, and their scrambled controls, ISIS 13043 and 13042, respectively. As shown in Table 3, the 3'-most nucleotide of each of these oligonucleotides was a 2'-deoxynucleotide (for ease of synthesis). All were tested against both vincristine and doxorubicin.

The results of these experiments are shown in FIGS. 1-4. The absorbance at 570 nm (Y-axis) is a measure of the viable cells remaining, thus low absorbance reflects cytotoxicity caused by the chemotherapeutic agent, vincristine or doxorubicin. C1 cells are not MRP overexpressers and thus are not resistant to vincristine or doxorubicin and are killed (open circles). T5 cells overexpress MRP, are drug resistant and are resistant to vincristine (closed circles).

Treatment with the antisense oligonucleotide ISIS 13041 (PS backbone, 8-deoxy gap) gave a virtually complete reversal of resistance to vincristine and over 50% reversal of resistance to doxorubicin (FIG. 2). This oligonucleotide compound is, therefore, highly preferred. ISIS 13039 (PS backbone, 12-deoxy gap) gave approximately 70% reversal of resistance to vincristine and 30% reversal of resistance to doxorubicin (FIG. 4). This compound is also highly preferred. ISIS 13038 (12-deoxy gap, mixed PS/PO backbone) gave approximately 40% reversal of resistance to vincristine, substantially more than control oligonucleotide 13044, but was very similar to control when tested with doxorubicin (FIG. 3). ISIS 13038 is preferred. ISIS 13040 (8-deoxy gap, PS/PO backbone) gave approximately 45% reversal of resistance to vincristine and doxorubicin, (FIG. 1), but the control, ISIS 13043, also gave partial reversal.

Reduction in MRP protein levels after double dose of oligo

Levels of MRP protein were assayed by immunoblot analysis after cells were treated with two doses of oligonucleotide. As shown in FIG. 5, MRP levels were reduced and held at low levels for at least 72 hours when treated with ISIS 13039 or ISIS 13041.

Antisense inhibition of H69AR tumor xenografts in nude mice

The effect of ISIS 7597 on growth of H69AR tumor xenografts in nude mice was examined both in comparison to and in combination with vincristine. Mice were implanted with tumor fragments and treated with ISIS 7597 (SEQ ID NO: 8, P=S, 25 mg/kg), vincristine, ISIS 7597 plus vincristine, or saline (control). Tumor size was measured weekly and the results are shown in FIG. 6. ISIS 7597 inhibited tumor growth by 30% on day 46 and by 38% on

day 50. This was better than vincristine alone (22% and 31% on days 46 and 50, respectively). The combination of ISIS 7597 and vincristine gave the greatest inhibition (63% and 70% on days 46 and 50, respectively).

Several preferred embodiments of this invention are exemplified in accordance with the following nonlimiting examples.

## EXAMPLES

### Example 1

#### Synthesis and characterization of oligonucleotides

Unmodified DNA oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.  $\beta$ -cyanoethyl-diisopropyl-phosphoramidites were purchased from Applied Biosystems (Foster City, Calif.). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-O-methyl phosphorothioate oligonucleotides were synthesized using 2'-O-methyl  $\beta$ -cyanoethyl-diisopropyl-phosphoramidites (Chemgenes, Needham Mass.) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. The 3'-base used to start the synthesis was a 2'-deoxyribonucleotide. 2'-O-propyl oligonucleotides were prepared by a slight modification of this procedure.

2'-Fluoro phosphorothioate oligonucleotides were synthesized using 5'-dimethoxytrityl-3'-phosphoramidites and prepared as disclosed in U.S. patent application Ser. No. 463,358, filed Jan. 11, 1990, and 566,977, filed Aug. 13, 1990, which are assigned to the same assignee as the instant application and which are incorporated by reference herein. The 2'-fluoro oligonucleotides were prepared using phosphoramidite chemistry and a slight modification of the standard DNA synthesis protocol: deprotection was effected using methanolic ammonia at room temperature.

2'-(2-methoxyethyl)-modified amides are synthesized according to Martin, P., *Helv. Chim. Acta* 1995, 78,486-504. For ease of synthesis, the last nucleotide was a deoxynucleotide. In most cases, 2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>-cytosines were 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers 2,2'-Anhydro [1-( $\beta$ -D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279M), diphenylcarbonate (90.0 g, 0.420M) and sodium bicarbonate (2.0 g, 0.024M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60° C. at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine

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2,2'-Anhydro-5-methyluridine (195 g, 0.81M), tris(2-methoxyethyl)borate (231 g, 0.98M) and 2-methoxyethanol (1.2L) were added to a 2L stainless steel pressure vessel and placed in a pre-heated oil bath at 160° C. After heating for 48 hours at 155°–160° C., the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5L) and extracted with 2×500 mL of saturated NaHCO<sub>3</sub> and 2×500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35° C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2×200 mL of saturated sodium bicarbonate and 2×200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl<sub>3</sub>. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane (4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144M) in CH<sub>3</sub>CN (700 mL) and set aside. Triethylamine (189 mL, 1.44M) was added to a solution of triazole (90 g, 1.3M) in CH<sub>3</sub>CN (1L), cooled to -5° C. and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred solution maintained at 0°–10° C., and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise,

## 16

over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1L) and the insoluble solids were removed by filtration. The filtrate was washed with 1×300 mL of NaHCO<sub>3</sub> and 2×300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141M) in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2×200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100° C. for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N<sup>4</sup>-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2×300 mL) and saturated NaCl (2×300 mL), dried over MgSO<sub>4</sub> and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N<sup>4</sup>-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N<sup>4</sup>-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1×300 mL) and saturated NaCl (3×300 mL). The aqueous washes were back-extracted with CH<sub>2</sub>Cl<sub>2</sub> (300 mL) and the extracts were combined, dried over MgSO<sub>4</sub> and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-hexylamincholesterol nucleosides were synthesized and incorporated into oligonucleotides according to Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651–3654.

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55° C. for 18 hours, the oligonucleotides were purified by precipitation twice out of 0.5M NaCl with 2.5 volumes ethanol. Analytical gel electrophoresis was



accomplished in 20% acrylamide, 8M urea, 45 mM Tris-borate buffer, pH 7.0. Oligodeoxynucleotides and phosphorothioate oligonucleotides were judged from electrophoresis to be greater than 80% full length material.

The relative amounts of phosphorothioate and phosphodiester linkages obtained by this synthesis were periodically checked by  $^{31}\text{P}$  NMR spectroscopy. The spectra were obtained at ambient temperature using deuterium oxide or dimethyl sulfoxide- $d_6$  as solvent. Phosphorothioate samples typically contained less than one percent of phosphodiester linkages.

#### Example 2

Selection and maintenance of multidrug resistant cell line H69AR cells

H69AR, a doxorubicin-resistant human small cell lung carcinoma cell line, was selected and maintained as described in Mirski et al. *Cancer Res.* 1987, 47, 2594-2598.

#### Example 3

Lipofection and oligonucleotide treatment of H69AR cells for analysis by whole cell ELISA

$1.5 \times 10^6$  cells were plated into 35 mm tissue culture wells and allowed to attach overnight. The cells were then washed twice with 3 ml of serum-free medium prior to lipofection. Oligonucleotides were added to a concentration of  $0.3 \mu\text{M}$  in 1 ml of serum-free medium in a polystyrene tube.  $10 \mu\text{l}$  of LIPOFECTIN<sup>TM</sup> (GIBCO-BRL) was then added and the mixture was vortexed. After ten minutes at room temperature, the DNA/LIPOFECTIN<sup>TM</sup> suspension was added to the cells and incubated for four hours at  $37^\circ\text{C}$ . After this incubation, 1 ml of 20% Hyclone serum in RPMI was added and left at  $37^\circ\text{C}$  overnight. The next day the suspension was removed and replaced with fresh medium. On the following day, the lipofection was repeated as before and the cells were harvested 48 hours after the second lipofection.

#### Example 4

Whole Cell ELISA of H69AR Cells after Oligonucleotide Treatment

Cells were harvested, counted and washed twice with PBS. Cells were resuspended at  $0.5\text{--}1 \times 10^5$  cells/ml in PBS and  $100 \mu\text{l}$  was plated in each well of an ELISA plate. Plates were dried overnight at  $37^\circ\text{C}$  and autocrosslinked twice in a Stratilinker (Stratagene, La Jolla, Calif.). Plates were rehydrated in TBST,  $200 \mu\text{l}/\text{well}$  for  $2 \times 5$  minutes. Wells were blocked for 1.5-2 hours at room temperature with  $200 \mu\text{l}$  TBST containing 5% NGS, 1% BSA. Primary antibody [ $50 \mu\text{l}$  of monoclonal antibody 3.186; Mirski et al. *Cancer Res.* 1989, 49, 5719-5724] diluted in blocking solution was added and plates were incubated for 1.5-2 hours in a humidified chamber at room temperature. Plates were washed  $3 \times 5$  minutes with  $200 \mu\text{l}$  TBST. Plates were incubated with  $50 \mu\text{l}$  secondary antibody diluted in blocking solution for 1-1.5 hours at room temperature in a humidified chamber. Plates were washed for with  $200 \mu\text{l}$  TBST,  $3 \times 5$  minutes. Color detection was by horseradish peroxidase [incubated with  $100 \mu\text{l}$  OPD/ $\text{H}_2\text{O}_2$ /citrate buffer ( $250 \mu\text{l}$  10 mg/ml OPD in methanol/ $25 \mu\text{l}$  3%  $\text{H}_2\text{O}_2$ / $24.8 \text{ ml}$  0.05M citrate pH 5)] in the dark for 30 minutes at room temperature, stop reaction with  $25 \mu\text{l}$  8N  $\text{H}_2\text{SO}_4$ , and read absorbance at 490 nm] or by alkaline phosphatase [incubate with  $50 \mu\text{l}$  substrate solution (1 PNPP tablet in 5 ml 50 mM  $\text{NaHCO}_3$ , pH 9.6, 1 mM  $\text{MgCl}_2$ ) for 30 minutes in humid chamber at room temperature, stop reaction with  $50 \mu\text{l}$  0.4M NaOH, read absorbance at 405 nm].

#### Example 5

RNA Analysis of H69AR Cells Treated with Antisense Oligonucleotides Specifically Hybridizable with MRP

$10 \times 10^6$  cells were plated per T75 flask and allowed to attach overnight. Cells were washed twice with serum-free medium before incubation with 6 ml of oligonucleotide/LIPOFECTIN<sup>TM</sup> suspension ( $0.3 \mu\text{M}$  oligonucleotide;  $10 \mu\text{l}$  LIPOFECTIN<sup>TM</sup> per ml of serum-free medium) at  $37^\circ\text{C}$  for 4 hours after which 6 ml of 20% Hyclone serum in RPMI was added and left overnight. Fresh medium was added the next day. On the following day polyadenylated RNA was isolated using a MICRO-FASTTRACK mRNA isolation kit (Invitrogen). The RNA was then separated by electrophoresis on a formaldehyde-agarose denaturing gel and then transferred to a nylon membrane (Zetaprobe, Biorad). The membrane was prehybridized in 50% formamide,  $5 \times \text{SSC}$ ,  $5 \times \text{Denhardt's}$  solution, 1% SDS and  $100 \mu\text{g}/\text{ml}$  sheared, denatured herring testis DNA for 4 hours at  $42^\circ\text{C}$ . The membrane was then hybridized overnight at  $42^\circ\text{C}$  with a 2.0 kb cDNA fragment of MRP labelled with [ $\alpha\text{-}^{32}\text{P}$ ]dCTP using a random prime kit (GIBCO/BRL). The blot was washed three times in 0.1% SDS and  $0.1 \times \text{SSC}$  for 20 minutes at  $52^\circ\text{C}$  and autoradiographed. Only in overloaded lanes was any RNA detectable as a faint band after oligonucleotide treatment with ISIS 7597 and 7598.

#### Example 6

Diagnostic assay for MRP-associated tumors using xenografts in nude mice

Tumors arising from MRP overexpression are diagnosed and distinguished from other tumors using this assay. A biopsy sample of the tumor is treated, e.g., with collagenase or trypsin or other standard methods, to dissociate the tumor mass.  $5 \times 10^6$  tumor cells are implanted in the inner thighs of two or more nude mice. Antisense oligonucleotide suspended in saline is administered to one or more mice by intraperitoneal injection three times weekly beginning on day 4 after tumor cell inoculation. Saline only is given to a control mouse. Oligonucleotide dosage is 25 mg/kg. Tumor size is measured and tumor volume is calculated on the eleventh treatment day. Tumor volume of the oligonucleotide-treated mice is compared to that of the control mouse. The volume of MRP-associated tumors in the treated mice are measurably smaller than tumors in the control mouse. Tumors arising from causes other than MRP overexpression are not expected to respond to the oligonucleotides targeted to the nucleic acids encoding MRP and, therefore, the tumor volumes of oligonucleotide-treated and control mice are equivalent.

#### Example 7

Detection of MRP overexpression

Oligonucleotides are radiolabeled after synthesis by  $^{32}\text{P}$  labeling at the 5' end with polynucleotide kinase. Sambrook et al., "Molecular Cloning. A Laboratory Manual," Cold Spring Harbor Laboratory Press, 1989, Volume 2, pg. 11.31-11.32. Radiolabeled oligonucleotide is contacted with tissue or cell samples suspected of MRP overexpression, such as tumor biopsy samples, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound oligonucleotide. A similar control is maintained wherein the radiolabeled oligonucleotide is contacted with normal cell or tissue sample under conditions that allow specific hybridization, and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide and is quantitated using a scintillation counter or other routine means. Comparison of the radioactivity remaining in the samples from normal and tumor cells indicates overexpression of MRP.



Radiolabeled oligonucleotides of the invention are also useful in autoradiography. Tissue sections are treated with radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to standard autoradiography procedures. A control with normal cell or tissue sample is also maintained. The emulsion, when developed, yields an image of silver grains over the regions expressing MRP, which is quantitated. The extent of MRP overexpression is determined by comparison of the silver grains observed with normal and tumor cells.

Analogous assays for fluorescent detection of MRP overexpression use oligonucleotides of the invention which are labeled with fluorescein or other fluorescent tags. Labeled DNA oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.  $\beta$ -cyanoethyl-diisopropyl phosphoramidites are purchased from Applied Biosystems (Foster City, Calif.). Fluorescein-labeled amidites are purchased from Glen Research (Sterling, Va.). Incubation of oligonucleotide and biological sample is carried out as described for radiolabeled oligonucleotides except that instead of a scintillation counter, a fluorescence microscope is used to detect the fluorescence. Comparison of the fluorescence observed in samples from normal and tumor cells indicates MRP overexpression.

#### Example 8

##### HeLa cell culture and oligonucleotide treatment

T5 and C1 HeLa cells were obtained and maintained as described in Canitrot et al. *Anti-Cancer Drugs* 1996, 7(suppl. 3), 93-99. Cells were treated with oligonucleotide in LIPOFECTIN™ essentially as described by Canitrot et al., except that a double treatment was used in some cases. For double treatment, cells were treated with oligonucleotide (500 nM) on day 1 and day 4 after plating. On day 5, cells were harvested for RNA and/or protein isolation. For MTT assay, chemotherapeutic agent was added on day 5 and the assay was performed on day 8.

#### Example 9

##### Measurement of MRP mRNA in HeLa cells

RNA quantitation was done by Northern blot analysis according to Canitrot et al., supra.

#### Example 10

##### Measurement of MRP protein in HeLa cells

Protein quantitation was done by immunoblot analysis according to Canitrot et al., supra.

#### Example 11

##### Chemosensitivity testing in HeLa cells

Drug sensitivity of transfected HeLa cells was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) -based cytotoxicity assay essentially as described in Cole et al. *Cancer Chemother. Pharmacol.* 1986, 17, 259-263 and Canitrot et al. *Anti-Cancer Drugs* 1996, 7(suppl. 3), 93-99. The MTT assay was performed 72 hours after chemotherapeutic agent was added to the cells.

#### Example 12

##### H69AR tumor xenograft studies

H69AR tumor fragments (approximately 25 mg each) were taken from two eighth-generation fragment xenografts in nude mice and implanted into recipient mice for study. Eighteen days later the animals were separated into three groups (7 or 8 mice per group) for treatment with either saline, vincristine, ISIS 7597 (SEQ ID NO: 8, P=S) or vincristine plus ISIS 7597. Treatment was 10 ml/kg daily for saline. For vincristine, three 1.5 mg/kg doses were given intraperitoneally on days 22, 36 and 46 for both the vincristine and vincristine+oligonucleotide groups. ISIS 7597 (25 mg/kg) was given daily by intravenous or intraperitoneal injection, beginning on day 19, for both the oligonucleotide and vincristine+oligonucleotide groups. Tumor size was measured weekly using calipers and converted to volume (cc), using the formula  $[\text{Volume} = \text{Length} \times \text{Width}^2 / 2]$ . Animal weights were also measured weekly. Treatment was terminated and animals sacrificed on day 50.

#### SEQUENCE LISTING

##### ( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 28

##### ( 2 ) INFORMATION FOR SEQ ID NO:1:

##### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGGGGCCGCA ACGCCGCCUG

2 0

##### ( 2 ) INFORMATION FOR SEQ ID NO:2:

##### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

-continued

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( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CGGGGCGCA ACGCCGCTG 20

( 2 ) INFORMATION FOR SEQ ID NO:3:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGTGATCGGG CCCGGTTGCT 20

( 2 ) INFORMATION FOR SEQ ID NO: 4:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCGGTGGCGC GGGCGGCGGC 20

( 2 ) INFORMATION FOR SEQ ID NO: 5:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGCCCCGGAG CGCCATGCCG 20

( 2 ) INFORMATION FOR SEQ ID NO: 6:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGGAGCCAT CGGCGCTGCA 20

( 2 ) INFORMATION FOR SEQ ID NO: 7:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGCACCCACA CGAGGACCGT 20



-continued

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( A ) LENGTH: 20  
( B ) TYPE: Nucleic Acid  
( C ) STRANDEDNESS: Single  
( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

A G G C C C T G C A G T T C T G A C C A

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 14:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 20  
( B ) TYPE: Nucleic Acid  
( C ) STRANDEDNESS: Single  
( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

C T C C T C C C T G G G C G C T G G C A

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 15:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 20  
( B ) TYPE: Nucleic Acid  
( C ) STRANDEDNESS: Single  
( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

A C C G G A T G G C G G T G G C T G C T

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 16:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 20  
( B ) TYPE: Nucleic Acid  
( C ) STRANDEDNESS: Single  
( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

C G C A T C T C T G T C T C T C C T G G

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 17:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 20  
( B ) TYPE: Nucleic Acid  
( C ) STRANDEDNESS: Single  
( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

C A G A A G C C C C G G A G C G C C A T

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 18:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 20  
( B ) TYPE: Nucleic Acid  
( C ) STRANDEDNESS: Single  
( D ) TOPOLOGY: Linear

-continued

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( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

G C C C C C G C C G T C T T T G A C A G 2 0

( 2 ) INFORMATION FOR SEQ ID NO: 19:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

G T G A T G C T G T T C G T G C C C C C 2 0

( 2 ) INFORMATION FOR SEQ ID NO: 20:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

C T C A C G G T G A T G C T G T T C G T 2 0

( 2 ) INFORMATION FOR SEQ ID NO: 21:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

C C C C C A G A C A G G T T C A C G C C 2 0

( 2 ) INFORMATION FOR SEQ ID NO: 22:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

C T G G C C C C C A G A C A G G T T C A 2 0

( 2 ) INFORMATION FOR SEQ ID NO: 23:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

G C C A G G C T C A C G C G C T G C T T 2 0

-continued

( 2 ) INFORMATION FOR SEQ ID NO: 24:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CACAGCCAGT TCCAGGCAGG

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 25:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCTGGGTCTT CACAGCCAGT

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 26:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CGGCGGGGGC ACGAACAGCA

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 27:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TCGTGGCCGC GTTCTCCCG

2 0

( 2 ) INFORMATION FOR SEQ ID NO: 28:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20
- ( B ) TYPE: Nucleic Acid
- ( C ) STRANDEDNESS: Single
- ( D ) TOPOLOGY: Linear

( i v ) ANTI-SENSE: Yes

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CGTGTTGCTC GTGCCCGCCC

2 0

What is claimed is:

1. An oligonucleotide comprising 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding mul-

65 tidrug resistance-associated protein and capable of inhibiting the expression of multidrug resistance-associated protein.

2. The oligonucleotide of claim 1 which is specifically hybridizable with mRNA encoding multidrug resistance-associated protein.

3. The oligonucleotide of claim 1 which specifically hybridizes with DNA encoding multidrug resistance-associated protein to form a triple stranded structure.

4. The oligonucleotide of claim 2 specifically hybridizable with a transcription initiation site, a translation initiation site, 5'-untranslated sequence, 3' untranslated sequence, coding sequence or an intron/exon junction of an mRNA encoding multidrug resistance-associated protein.

5. A pharmaceutical composition comprising an oligonucleotide of claim 1 and a pharmaceutically acceptable carrier.

6. The oligonucleotide of claim 1 wherein at least one of the intersugar linkages between nucleotides of the oligonucleotide is a phosphorothioate linkage.

7. The oligonucleotide of claim 1 wherein at least one of the nucleotides is modified at the 2' position.

8. The oligonucleotide of claim 7 wherein the nucleotide modification is 2'-O-methyl, 2'-O-propyl, 2'-methoxyethoxy or 2'-fluoro.

9. The oligonucleotide of claim 8 which is a chimeric oligonucleotide.

10. The oligonucleotide of claim 9 which is a gapped oligonucleotide.

11. The oligonucleotide of claim 1 comprising SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28.

12. An oligonucleotide comprising 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding multidrug resistance-associated protein and capable of inhibiting the expression of multidrug resistance-associated protein, wherein said oligonucleotide comprises at least one 2'-methoxyethoxy modification.

13. The oligonucleotide of claim 12 which is a chimeric oligonucleotide.

14. The oligonucleotide of claim 13 which is a gapped oligonucleotide.

15. An oligonucleotide of claim 12 specifically hybridizable with a transcription initiation site, a translation initiation site, 5'-untranslated sequence, 3' untranslated sequence, coding sequence or an intron/exon junction of an mRNA encoding multidrug resistance-associated protein.

16. A pharmaceutical composition comprising an oligonucleotide of claim 12 and a pharmaceutically acceptable carrier.

17. An oligonucleotide of claim 12 wherein at least one of the intersugar linkages between nucleotides of the oligonucleotide is a phosphorothioate linkage.

18. An oligonucleotide of claim 12 comprising SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28.

19. An oligonucleotide comprising SEQ ID NO: 8 and at least one 2' methoxyethoxy modification.

20. The oligonucleotide of claim 19 which is a chimeric oligonucleotide.

21. The oligonucleotide of claim 20 which is a gapped oligonucleotide.

22. A method of inhibiting the synthesis of multidrug resistance-associated protein in a cell or tissue comprising

contacting a cell or tissue with an oligonucleotide comprising 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding multidrug resistance-associated protein and capable of modulating the expression of multidrug resistance-associated protein.

23. A method of inhibiting the synthesis of multidrug resistance-associated protein in a cell or tissue comprising contacting a cell or tissue with an oligonucleotide comprising an oligonucleotide comprising SEQ ID NO: 8 and at least one 2' methoxyethoxy modification.

24. The method of claim 23 wherein the oligonucleotide is a chimeric oligonucleotide.

25. The method of claim 23 wherein the oligonucleotide is a gapped oligonucleotide.

26. A method of treating an animal suspected of having a condition which is characterized by overexpression of multidrug resistance-associated protein comprising administering to said animal a therapeutically effective amount of an oligonucleotide having 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding multidrug resistance-associated protein and capable of inhibiting the expression of multidrug resistance-associated protein.

27. The method of claim 26 wherein the oligonucleotide is administered in conjunction with a chemotherapeutic drug treatment for cancer.

28. The method of claim 27 wherein the condition is a multidrug-resistant cancer.

29. The method of claim 27 wherein the multidrug-resistant cancer is small-cell lung cancer.

30. A method for improving the efficacy of a chemotherapeutic drug treatment of a disease, said method comprising administering in conjunction with a chemotherapeutic drug treatment an oligonucleotide comprising 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding multidrug resistance-associated protein and capable of inhibiting the expression of multidrug resistance-associated protein.

31. The method of claim 30 wherein the disease is cancer.

32. A method for preventing the development of multidrug resistance during a chemotherapeutic drug treatment of a disease, said method comprising administering in conjunction with a chemotherapeutic drug treatment an oligonucleotide comprising 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding multidrug resistance-associated protein and capable of inhibiting the expression of multidrug resistance-associated protein.

33. The method of claim 32 wherein the disease is cancer.

34. A method of treating an animal suspected of having a condition which is characterized by leukotriene production comprising administering to said animal a therapeutically effective amount of an oligonucleotide having 8 to 30 nucleotides specifically hybridizable with a nucleic acid encoding multidrug resistance-associated protein and capable of inhibiting the expression of multidrug resistance-associated protein.

35. The method of claim 34 wherein the condition is an inflammatory condition.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 5,801,154

DATED : September 1, 1998

INVENTOR(S) : Baracchini, et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, item [57],

**ABSTRACT**, at line 5, delete "referred  
and insert --preferred--.

Signed and Sealed this

Nineteenth Day of January, 1999

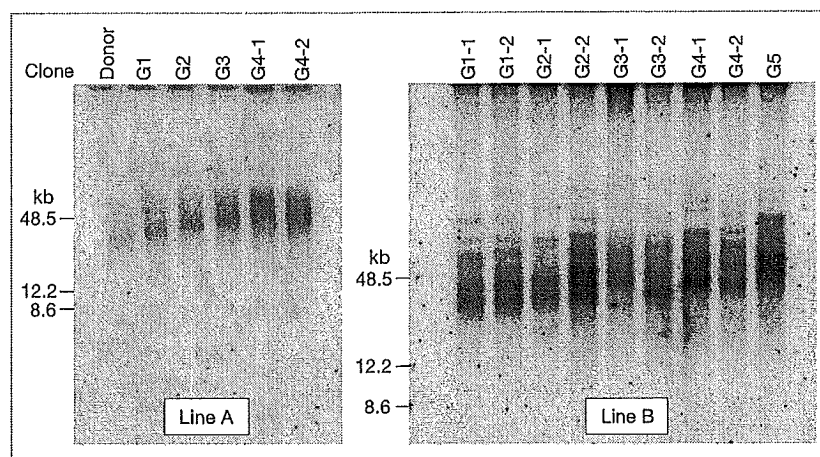
Attest:



Attesting Officer

*Acting Commissioner of Patents and Trademarks*





**Figure 1** Telomere lengths in successive generations (G1–G5) of mice cloned from cumulus cells. Southern-blot analysis of terminal restriction-enzyme-cut fragments in five sequential generations shows that telomeres do not undergo incremental erosion in successive clonal generations. Genomic DNA isolated from peripheral-blood lymphocytes taken from representative animals from each generation was digested with the restriction enzyme *HinfI*, resolved on a pulse field gel, transferred to a solid support and probed with a 5'-<sup>32</sup>P-labelled (T<sub>4</sub>AG)<sub>3</sub> oligonucleotide. Peripheral blood lymphocytes were sampled on the same day. Ages of mice (in months) were: in line A, donor, 18; G1, 16; G2, 14; G3, 12; G4, 9; G5, 9; in line B, G1, 15.5; G2, 13; G3, 11; G4, 9; G5, 7. Suffix numbers (G4-1 and G4-2, for example) identify different pups of each generation.

was repeated with cumulus cells from adult G1 mice as nucleus donors to produce the next clonal generation, G2, and so on. Table 1 summarizes the results obtained following the reconstruction of 3,920 enucleated oocytes.

Previously, about 2% of enucleated oocytes receiving a cumulus cell nucleus developed to live-born pups<sup>1</sup>. This value is comparable to the cloning efficiency for G1 in lines A (1.5%) and B (4.2%). However, the success rate dropped in successive cloned generations: line A did not produce a G5 clone from 670 reconstructed oocytes; in line B, the only live-born G6 clone was cannibalized by her foster mother, thereby terminating the line. Mouse lines A and B therefore represent totals of 9 and 26 clones from their respective donors. Placental size was consistently in the range previously reported for cloned mice<sup>2</sup> and did not increase in successive generations (data not shown).

Do sequentially cloned mice show signs of accelerated ageing? We assessed the behaviour of these mice and determined telomere lengths to assess organismal and cellular measures of ageing, respectively. We evaluated learning ability in the Morris water maze and Krushinsky tests, as well as strength and agility, and also used other

assays designed to monitor signs of premature ageing, such as a decline in activity in the home cage and loss of coordination<sup>4</sup>. All cloned mice were, by these criteria, normal compared with age-matched controls (data not shown); the G5 mouse is alive and healthy at 1.5 years.

We measured telomere length in peripheral blood lymphocytes of clones G1–G6 by Southern-blot analysis of terminal restriction-enzyme-digested fragments (Fig. 1) and found no evidence of shortened telomeres in the cloned mice. In fact, our results show that the telomeres lengthen with each generation. As representative animals of each generation were sampled simultaneously, we cannot rule out an age-related contribution to this increase (with younger mice having longer telomeres). In addition, long telomeres in mice are optimally studied by means of different assays such as quantitative fluorescence *in situ* hybridization<sup>5</sup>. We have detected telomerase activity in cumulus cells (data not shown); it is therefore possible that telomeres in these cells are unusually long, resulting in offspring with concomitantly longer telomeres.

Shortened<sup>6</sup> and lengthened<sup>7</sup> telomeres have been reported in cloned livestock but, unlike ours, those experiments involved only a single round of cloning. Our results

on sequentially cloned mice verify that telomere shortening is not a necessary outcome of the cloning process<sup>8</sup>. However, as only 1–2% of reconstructed oocytes yield live-born clones, the possibility of selection for donor nuclei with the longest telomeres cannot be excluded. Further investigation is required into the consequences of nuclear transfer on telomere length and lifespan.

Teruhiko Wakayama\*†, Yoichi Shinkai‡, Kellie L. K. Tamashiro\*, Hiroyuki Niida§, D. Caroline Blanchard||, Robert J. Blanchard||, Atsuo Ogura¶, Kentaro Tanemura¶, Makoto Tachibana‡,

Anthony C. F. Perry#, Diana R. Colgan#, Peter Mombaerts#, Ryuzo Yanagimachi\*

\*Department of Anatomy and Reproductive Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822, USA

‡Department of Cell Biology, Institute for Virus Research, Kyoto University, Sakyo-Ku, Kyoto 606-8507, Japan

§Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720, USA

||Bekesy Laboratory of Neurobiology, University of Hawaii, Honolulu, Hawaii 96822, USA

¶Department of Veterinary Science, National Institute of Infectious Disease, Tokyo 162-8640, Japan

#The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

†Present address: The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

e-mail: wakayama@mail.rockefeller.edu

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### Gene expression

## Total silencing by intron-spliced hairpin RNAs

Post-transcriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism inherent in many life-forms, can be induced in plants by transforming them with either antisense<sup>1</sup> or co-suppression<sup>2</sup> constructs, but typically this results in only a small proportion of silenced individuals. Here we show that gene constructs encoding intron-spliced RNA with a hairpin structure can induce PTGS with almost 100% efficiency when directed against viruses or endogenous

**Table 1** Effect of sequential cloning on full-term development

Line	G1	G2	G3	G4	G5	G6	Total
A	2/131 (1.5)	1/228 (0.4)	1/263 (0.4)	5/238 (2.1)	0/670 (0)	-	9/1,530 (0.6)
B	4/96 (4.2)	7/351 (2.0)	5/352 (1.4)	6/286 (2.1)	3/581 (0.5)	1/724 (0.1)	26/2,390 (1.1)

Successive generations are represented as G1, G2 and so on for two independent mouse lines, A and B. The number of pups born live after cumulus-cell nuclear transfer is compared to successfully reconstructed oocytes (pups/oocytes), with the corresponding percentages in parentheses. Significant  $\chi^2$  comparisons were derived for G4 and G5 from line A, G1 and G5, G6 from line B, and G2, G3, G4 versus G6 from line B ( $P < 0.05$ ).

genes. These constructs could prove valuable in reverse genetics, genomics, engineering of metabolic pathways and protection against pathogens.

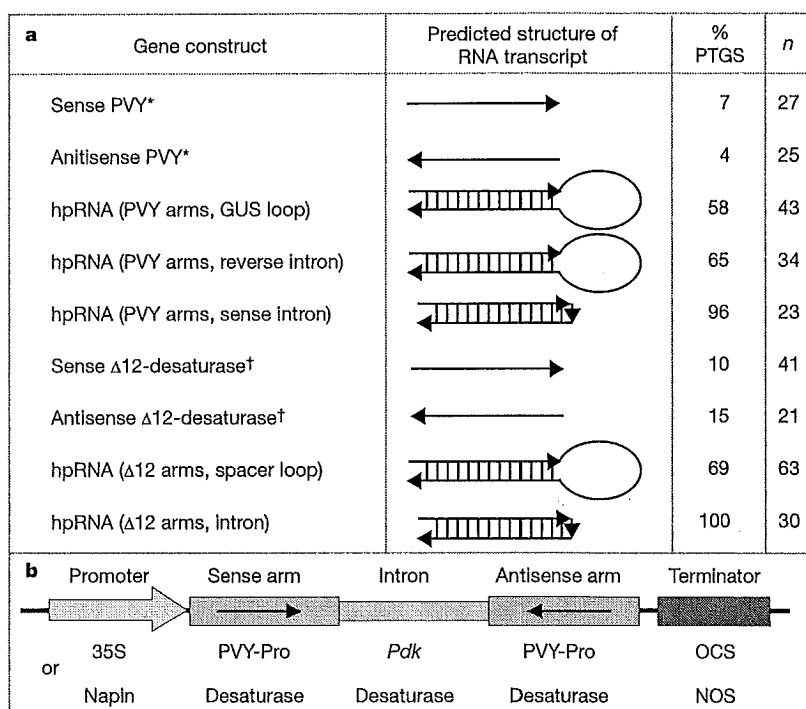
Induction of PTGS by co-suppression and antisense methods that target the *Nia*-protease (*Pro*) gene sequence of potato virus Y (PVY)<sup>3</sup> cause silencing in 7% and 4% of independent transformants, respectively; induction of PTGS in these tobacco plants (*Nicotiana tabacum*) manifests as immunity<sup>3,4</sup> to the virus.

Using principles we developed for silencing constructs that express double-stranded RNA and inverted-repeat RNAs<sup>3</sup>, we made a construct encoding a single self-complementary hairpin RNA (hpRNA) of the *Pro* sequence. The construct contains sense and antisense *Pro* sequences flanking an 800-nucleotide spacer fragment derived from the *uidA* (GUS) gene (Fig. 1a). About 60% (25/43) of the plants that are transformed with this construct, many of which contained a single transgene copy, were immune to the virus. The spacer fragment contributed to the stability of the perfect inverted-repeat sequences, but it was not required for the specificity of the PTGS (Fig. 1a).

To test the effect of removing the loop region of hpRNA, we replaced the spacer with an intron sequence (Fig. 1a, b). The intron sequence provides stability to the DNA, but is spliced out during pre-mRNA processing<sup>5</sup> to produce loopless hpRNA. As a control, we made a sister construct in which the intron sequence was inserted in the reverse, non-splicing, orientation. When transformed into tobacco, 22 of 34 (65%) reverse-intron plants were immune, a similar frequency to plants transformed with the GUS spacer construct. Amazingly, we found that 22 of 23 plants transformed with the construct containing the functional intron were immune to the virus.

To test whether this enhancement by the sense-intron construct was a general phenomenon, we made two hpRNA constructs against the endogenous  $\Delta 12$ -desaturase (*Fad2*) gene of *Arabidopsis*, which catalyses the conversion of oleic to linoleic acid in the seed<sup>6,7</sup>; one construct contained an intron and the other a non-intron spacer region. We found that 69% (44/63) of the transgenic plants with the non-intron spacer region construct showed PTGS of the  $\Delta 12$ -desaturase gene, but that 100% (30/30) of plants transformed with the intron construct showed silencing of this gene.

How does the presence of this intron enhance silencing efficiency? The process of intron excision from the construct by the spliceosome might help to align the complementary arms of the hairpin in an environment favouring RNA hybridization, promoting the formation of a duplex. Alternatively, splicing may transiently increase



**Figure 1** Efficiency of induction of post-transcriptional gene silencing (PTGS) by different gene constructs and the predicted structure of RNA transcribed from the transgenes. **a**, PTGS efficiency measured for potato virus (PVY) and  $\Delta 12$ -desaturase genes as the percentage of independent transgenic plants immune to PVY and the percentage of plants with enzyme activity reduced by more than 20% compared with wild type, respectively. In the predicted structures of RNA transcripts, right- and left-pointing arrows represent sense and antisense orientation of sequences, respectively; small vertical arrows represent splice-junction sequences remaining after the intron has been spliced out. Vertical lines in the predicted structures indicate duplex formation. Asterisks, data from ref. 3; daggers, data from ref. 7; hpRNA, hairpin RNA; n, number of independent transformants; GUS,  $\beta$ -glucuronidase. **b**, Design of Intron-containing hairpin constructs. OCS, octopline synthase; NOS, nopaline synthase.

the amount of hairpin RNA by facilitating, or retarding, the hairpin's passage from the nucleus, or by creating a smaller, less nuclease-sensitive loop.

Our PVY constructs contained intron-2 from the *Pdk* gene of *Flaveria*<sup>8</sup>, whereas the  $\Delta 12$ -desaturase construct contained intron-1 from the *Arabidopsis Fad2* gene (Fig. 1b). PVY constructs were controlled by the constitutive CaMV35S (ref. 9) promoter and produced hpRNA containing the PVY coding-region sequence (700 nucleotides); the desaturase gene construct used the seed-specific napin promoter<sup>10</sup> to produce hpRNA representing 120 nucleotides of the 3'-untranslated region of the  $\Delta 12$ -desaturase gene.

We believe that constructs encoding intron-hpRNA should efficiently induce PTGS for a wide range of genes in a variety of circumstances and could become as useful to plant biology as RNAi<sup>11,12</sup> is to the study of nematodes and *Drosophila*. The transgene design might also have applica-

tion in organisms in which RNAi has been obtained by injection of double-stranded RNA.

Neil A. Smith, Surinder P. Singh, Ming-Bo Wang, Peter A. Stoutjesdijk, Allan G. Green, Peter M. Waterhouse  
CSIRO Plant Industry, Canberra, ACT 2601, Australia

e-mail: peter.waterhouse@pi.csiro.au

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# Exhibit 10

*The Plant Journal* (2001) 27(6), 581–590

## TECHNICAL ADVANCE

# Construct design for efficient, effective and high-throughput gene silencing in plants

S. Varsha Wesley<sup>1,†</sup>, Christopher A. Helliwell<sup>1,†</sup>, Neil A. Smith<sup>1,†</sup>, MingBo Wang<sup>1</sup>, Dean T. Rouse<sup>1</sup>, Qing Liu<sup>1</sup>, Paul S. Gooding<sup>1</sup>, Surinder P. Singh<sup>1</sup>, David Abbott<sup>1</sup>, Peter A. Stoutjesdijk<sup>1</sup>, Simon P. Robinson<sup>1</sup>, Andrew P. Gleave<sup>2</sup>, Allan G. Green<sup>1</sup> and Peter M. Waterhouse<sup>1,\*</sup>

<sup>1</sup>CSIRO Plant Industry, PO Box 1600 Canberra ACT 2601, Australia, and

<sup>2</sup>HortResearch, Mt Albert Research Centre, Private Bag 92 169, Auckland, New Zealand

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\*For correspondence (fax +61 262465000; e-mail peterw@pi.csiro.au).

†These authors contributed equally to this work.

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## Summary

Post-transcriptional silencing of plant genes using anti-sense or co-suppression constructs usually results in only a modest proportion of silenced individuals. Recent work has demonstrated the potential for constructs encoding self-complementary 'hairpin' RNA (hpRNA) to efficiently silence genes. In this study we examine design rules for efficient gene silencing, in terms of both the proportion of independent transgenic plants showing silencing, and the degree of silencing. Using hpRNA constructs containing sense/anti-sense arms ranging from 98 to 853 nt gave efficient silencing in a wide range of plant species, and inclusion of an intron in these constructs had a consistently enhancing effect. Intron-containing constructs (ihpRNA) generally gave 90–100% of independent transgenic plants showing silencing. The degree of silencing with these constructs was much greater than that obtained using either co-suppression or anti-sense constructs. We have made a generic vector, pHANNIBAL, that allows a simple, single PCR product from a gene of interest to be easily converted into a highly effective ihpRNA silencing construct. We have also created a high-throughput vector, pHELLSGATE, that should facilitate the cloning of gene libraries or large numbers of defined genes, such as those in EST collections, using an *in vitro* recombinase system. This system may facilitate the large-scale determination and discovery of plant gene functions in the same way as RNAi is being used to examine gene function in *Caenorhabditis elegans*.

**Keywords:** PTGS, RNAi, genomics, vector, ihpRNA, Gateway.

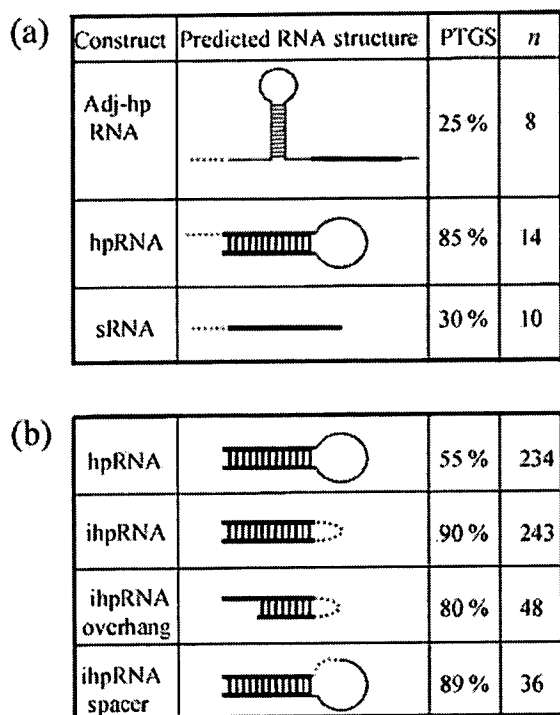
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## Introduction

The ultimate goal of current genome projects is to identify the biological function of every gene in the genome. Whole genomes of several organisms (including *Arabidopsis*, <http://www.arabidopsis.org>), have been completely sequenced, providing a wealth of information. The functions of some of the genes have been identified directly by the appropriate assay, or have been inferred by homology to genes of known function in other organisms. Loss-of-function mutants, from insertional mutagenesis or transposable elements, have also been very informative about the role of some of

these genes (AzpirozLeehan and Feldmann, 1997; Martienssen, 1998), particularly in the large-scale analysis of the yeast genome (Ross-Macdonald *et al.*, 1999). However, the functions of a large proportion of genes remain unknown.

Injection or ingestion of dsRNA into nematodes can trigger specific RNA degradation, in a process known as RNA-interference (RNAi; Fire *et al.*, 1998). This process facilitates targeted post-transcriptional gene silencing (PTGS) and has recently been harnessed to study the function of over 4000 genes on chromosomes I and III



**Figure 1.** The predicted RNA structure and efficacy of gene-silencing constructs.

(a) Three constructs, controlled by Ubi1 promoter, silencing GUS in rice. Thick lines indicate a 560 nt GUS sequence; grey lines indicate non-GUS sequences; dashed grey lines indicate intron-junction sequences left after splicing; and short lines within the stem of hairpin structures indicate base pairing. Numbers in PTGS column indicate the percentage of plants showing GUS silencing; *n* = number of plants in each treatment.

(b) Silencing efficacy of four different construct types with sequences as depicted in (a), except the thick lines in hpRNA and ihpRNA represent the various different target sequences in Table 1; and the thick lines in iphRNAoverhang and iphRNAspacer represent PVY Pro sequences. The percentage PTGS of hpRNA and ihpRNA, and iphRNAoverhang and iphRNA spacer, are the average percentage silencing of these types of constructs reported in Table 1 and the percentage of plants showing immunity to PVY, respectively.

in *Caenorhabditis elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). We discovered that transgenes designed to express double-stranded or single-stranded self-complementary (hairpin) RNA have a similar post-transcriptional silencing effect in plants (Wang and Waterhouse, 2000; Waterhouse *et al.*, 1998) and that, in at least two examples, almost 100% of plants transformed with an intron-containing hairpin RNA construct showed silencing (Smith *et al.*, 2000). These results led us to ask whether hpRNA technology might be exploited for gene discovery in plants. We have sought to design and evaluate generic intron-hpRNA constructs that might enable plant gene-discovery studies on a scale that matches those in nematodes.

## Results

### Location of silence-inducing sequences in hairpin RNA constructs

Constructs encoding RNAs with regions of self-complementarity efficiently induce gene silencing. We have previously shown that the sequences in the duplex stem in hpRNAs direct gene silencing (Smith *et al.*, 2000; Waterhouse *et al.*, 1998), whereas the results of Hamilton *et al.* (1998) suggest that single-stranded RNA sequences adjacent to a potential hairpin-forming structure give sequence specificity to silencing. The latter arrangement (adj-hpRNA) could be easily incorporated into gene-silencing vectors as the sequence encoding the hairpin RNA could be generic to the vector, while the specificity of the silencing would be accomplished by simply inserting a single copy of target gene sequence. In contrast, hpRNA constructs require two copies of the target sequence in an inverted-repeat orientation, in order to produce duplex RNA. To compare the relative efficacy of the designs, various GUS-silencing constructs, under the control of the Ubi1 promoter and associated intron, were made (Figure 1a) and super-transformed into GUS-expressing rice. Histochemical staining of the transformed plants showed that the adj-hp RNA construct gave no higher frequency of silenced lines than conventional co-suppression (sRNA), but the hpRNA construct gave many more silenced lines (Figure 1a). This suggested that the hpRNA was the design of choice.

Examination of the stained rice Ubi-hpGUS plants and a similar 35S-hpGUS construct in tobacco (Figure 2f) showed that the silencing was evenly distributed throughout the plant. Analysis of RNAs in the tobacco plants for the presence of GUS-derived small (~21 nt) RNAs showed a perfect correlation between the presence of these molecules and the presence of the 35S-hpGUS construct and silencing of the target GUS gene (Figure 3). This confirms that the silencing was due to PTGS; such small RNAs are a hallmark of PTGS (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001)

### Intron-spliced hpRNA vectors

We have found that it is necessary to include a spacer region between the arms of hpRNA constructs for stability of the inverted repeat DNA in *Escherichia coli*. However, replacing the spacer (loop) region of hpRNA constructs with a functional intron sequence increases the proportion of independent silenced lines recovered from approximately 50 to about 100% (Smith *et al.*, 2000). In these experiments, the targets were potato virus Y (PVY) and the *FAD2* Δ12-desaturase gene of *Arabidopsis*. The constructs were designed such that their pre-mRNA should splice to

form hpRNAs with small loops (Figure 4). The PVY construct should give an hpRNA comprising a 730 nt stem and a 6 nt loop; the hpRNA from the  $\Delta 12$ -desaturase construct should contain a 120 nt stem and a 21 nt loop. Although the PVY construct contained only two and four bases of original exon sequence 5' and 3' of the intron, respectively, the intron was still functional. RT-PCR and sequencing of transgene mRNA in plants containing the *HindIII* intron fragment (Figure 4) showed that the intron was cleaved out, leaving the predicted splice junction (data not shown).

The  $\Delta 12$ -desaturase result (100% of independent plants showing silencing despite having a 21 nt loop in the hpRNA) showed that the intron-enhanced silencing was not solely due to the tightness of the hairpin loop. Therefore we wondered whether this could be exploited to make a generic intron-spliced hpRNA (ihpRNA) vector into which the gene, or gene fragment, of choice could be directionally cloned to make sense and anti-sense arms. The vector pHANNIBAL (Figures 4 and 5), and a sister vector, pKANNIBAL (with bacterial ampicillin and kanamycin resistance genes, respectively), were designed so that a PCR fragment could be inserted in the sense orientation into the *XhoI*.*EcoRI*.*KpnI* polylinker and in the anti-sense orientation in the *Clal*.*HindIII*.*BamHI*.*XbaI* polylinker. This may be accomplished either by two separate PCR reactions with the appropriate single sites introduced with each primer, or by a single PCR using primers each introducing two restriction sites (e.g. primer 1, *XbaI*.*XhoI*.xxx; primer 2, *Clal*.*KpnI*.xxx). The construct will produce an hpRNA with a loop of 30–50 bases depending on which restriction sites are used to insert the targeting gene sequences.

The efficacy of pHANNIBAL was tested in *Arabidopsis* targeting the pigment biosynthesis gene chalcone synthase (*CHS*); the ethylene signalling gene *EIN2*; and the flowering repression gene *FLC1*. These genes were chosen because their mutant alleles have been reported in *Arabidopsis* to give distinct phenotypes. The *tt4* (CS85) EMS mutant (Koornneef *et al.*, 1990) produces inactive CHS, resulting in reduced production of flavonoid pigments in both the stem and seed coat. The mutant *ein2* (Alonso *et al.*, 1999) is insensitive to ethylene and grows well on media containing 1-aminocyclopropane-1-carboxylic acid, whereas wild-type plants develop a very stunted appearance when grown on such media. The mutant *flc1* (Amasino *et al.*, 2000) flowers earlier than wild-type *Arabidopsis*.

A 741 nt piece of CHS coding region was amplified from *A. thaliana* (Landsberg *erecta*) using primers that added an *XhoI* and a *KpnI* site on the ends of one product and an *XbaI* and *BamHI* site on the ends of the other product. These two amplification products were then directionally cloned into pHANNIBAL (Figure 5).

Similar cloning strategies were adopted for a 600 nt sequence from *EIN2*, and both a 650 nt and a 400 nt sequence from *FLC1*. As controls, sense and anti-sense constructs of CHS and an anti-sense construct of *FLC1* were also generated. All the constructs were subcloned into the binary vector pART27 and transformed into *Arabidopsis*.

Only two of the 19 plants transformed with the CHS co-suppression construct, and none of the 25 plants transformed with the CHS anti-sense construct, showed any obvious evidence of silencing. Whereas over 90% (21 of 23) of the plants transformed with the CHS-HANNIBAL constructs showed pronounced silencing (Table 1). The seed colours of most of these lines were virtually indistinguishable, to the naked eye, from seed of the *tt4*(CS85) mutant (Figure 2a). Examination of the seed under a light microscope revealed that the degree of pigmentation was generally uniform in the cells of the coat of an individual seed, and among seeds of the same line (Figure 2b,c). There was a perceptible difference in the levels of pigmentation between the different lines. The relative flavonoid content of seed from three lines selected to span the range of seed colour in the plant lines transformed with CHS-HANNIBAL, and from the co-suppression line giving the lightest coloured seed were 7, 23, 47 and 75%, respectively. The *tt4* (CS85) and wild-type seed had values of 0 and 100%, respectively.

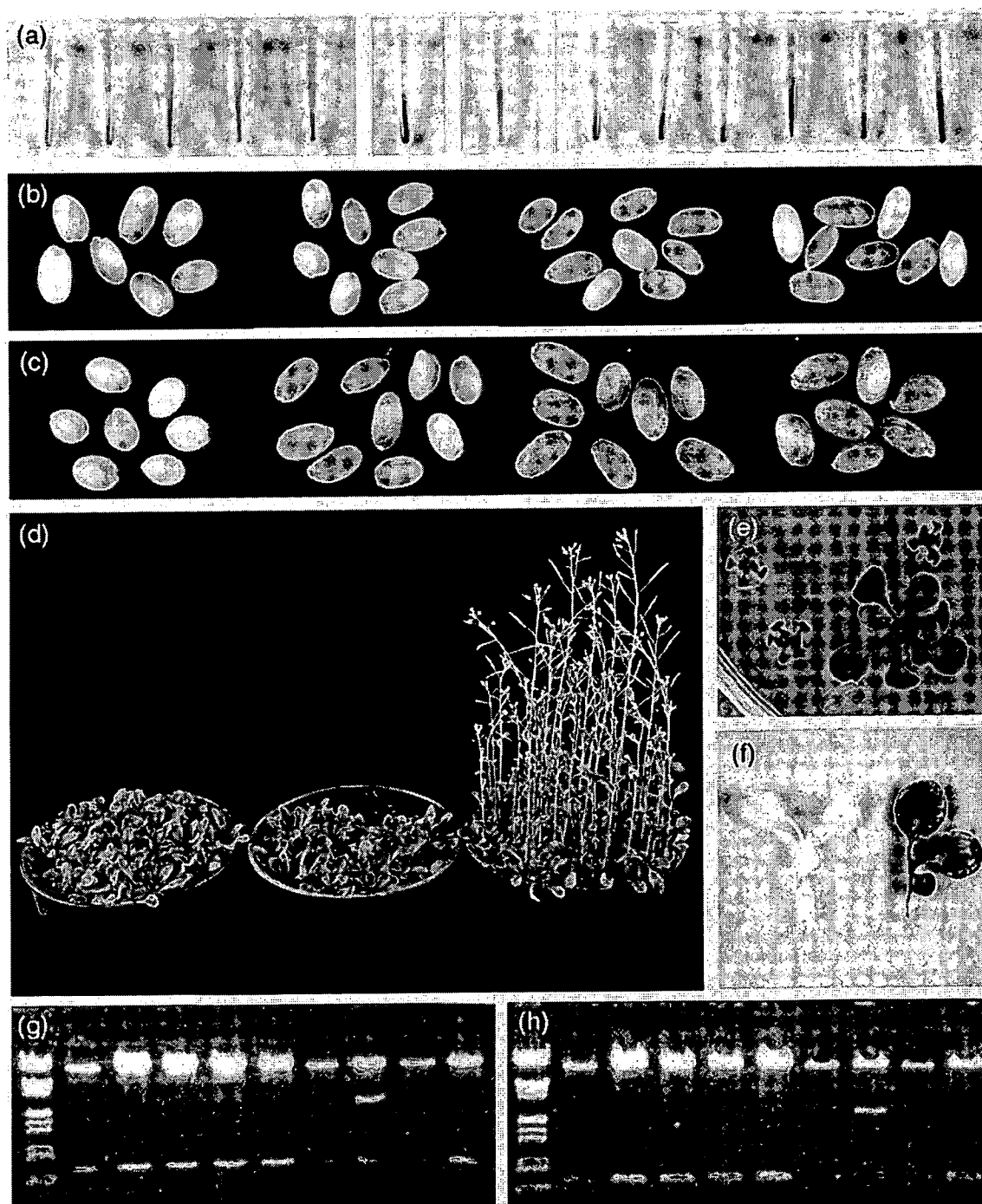
Sixty-four independent lines transformed with the *EIN*-HANNIBAL construct were obtained. The progeny from 42 of the lines showed Mendelian segregation for normal and stunted growth when grown on ACC medium, whereas all wild-type plants showed a stunted morphology on this medium (Table 1; Figure 2e).

The transgenic progeny of every one of the 31 independent plants transformed with the *FLC1*-HANNIBAL constructs flowered earlier and made fewer leaves, prior to flowering, than wild-type plants (Table 1; Figures 2d and 6). The transgenic progeny from the majority of the plants transformed with the *FLC1* anti-sense construct had similar leaf numbers and flowering times to those of wild-type plants. The flowering time and leaf number provided an easy measurement of the degree of silencing in individual lines. Only two of the 31 *FLC1*-HANNIBAL plant lines did not flower in less than 25 days after germination, whereas only two anti-sense plant lines flowered in less than 30 days. This suggests that ihpRNA constructs not only give an increased proportion of silenced transformants than anti-sense constructs, but also give more profound levels of silencing. However, even the most profoundly silenced *FLC1*-HANNIBAL line flowered 1 day later than the *flc* mutant, suggesting that it was not quite the equivalent of a null allele.

Collectively, pHANNIBAL-based constructs (which are driven by the constitutive 35S promoter) have been

made for five different genes (*CHS*, *EIN2*, *FLC1*, PVY-Pro and polyphenol oxidase – *PPO*). Similar intron-containing constructs targeting seed specifically against two different genes ( $\Delta 12$ - and  $\Delta 9$ -desaturase) in *Arabidopsis* and/or cotton have been tested for their silencing efficiency. Intron-less hpRNA, anti-sense and co-suppres-

sion constructs have also been used in many of these gene/host combinations. The results are summarized in Table 1. The ihpRNA constructs were effective, with arm lengths ranging from 98 to 853 nt, giving 66–100% (average 90%) independent silenced transformants. Intron-free hpRNA constructs gave 48–69% (average 58%) silenced



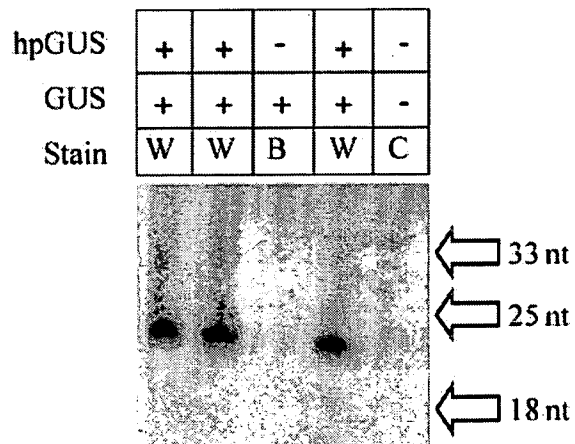
transformants, and conventional co-suppression or anti-sense constructs gave 0–30% (average 13 and 12%, respectively) silenced transformants. The intron-spliced or intron-free hpRNA constructs were effective when targeted against the coding, 5' untranslated or 3' untranslated regions of the mRNA. Taken together, these results indicate that ihpRNA constructs consistently give the most efficient silencing under a wide range of conditions.

#### Effect of intron location and unbalanced arms in pHANNIBAL

Intron-spliced hpRNA constructs appear to give a higher proportion of silenced transformants than intron-free hpRNA constructs. One explanation for this might be that the process of intron-splicing aligns the arms of the hpRNA, facilitating their duplex formation in the spliceosome complex, whereas the arms of hpRNAs have to find their self-complementarity by random, but tethered, collisions. If there is a threshold of duplex RNA required for PTGS in plants, then facilitating more efficient duplex RNA formation from ihpRNA might raise the level in low transgene-expressing plants such that PTGS is enabled. Similar levels of transcription of non-spliced hpRNA might produce lower steady-state levels of duplex RNA that are insufficient for PTGS. The same threshold theory could also be applied for the tighter loop of ihpRNA, giving more nuclease-stable and higher steady-state duplex RNA levels than the larger looped hpRNA. To test the validity of these possibilities, a construct was made in which a spacer region was inserted between one of the arms and the intron in a PVY ihpRNA construct (Figure 1b). This spacer region should impede alignment of the arms during the splicing process and produce a spliced hpRNA with a large loop. When plants transformed with the construct were challenged with PVY, 32 out of 36 independent transformants were immune to the virus. This suggests that the majority of the intron-enhanced silencing efficiency is not due to better alignment of the RNA arms or by presence of a tighter ssRNA loop. It may also explain why the GUS–hpRNA construct so efficiently silenced GUS in

the rice plants reported in Figure 1(a), as this construct contained an intron in the 5' untranslated leader sequence of the ubiquitin promoter.

A common feature of our hpRNA constructs has been the use of matched-length arms. These constructs should produce hpRNA with only a few unpaired 5' nucleotides. If pHANNIBAL is to be used as a generic vector for inserts from gene libraries, occasionally a restriction site within the PCR fragment will be common to the one used to clone into the polylinker. This will sometimes lead to the unintended construction of an hpRNA with unmatched arm length. To investigate whether this was an important attribute, a pHANNIBAL construct was made (using the



**Figure 3.** Detection of short (~22 nt) GUS-derived RNAs in tobacco plants showing hpRNA-mediated GUS silencing.

Total RNA (20 µg each), isolated from tobacco plants showing independent segregation of a 35S GUS transgene and a 35S hpGUS transgene, was separated in a 15% denaturing polyacrylamide gel, blotted onto a Hybond-N membrane and hybridized with *in vitro*-transcribed <sup>32</sup>P-labelled GUS RNA. The presence (+) or absence (–) of the target GUS and/or the 35S hpGUS transgene in the plant, from which the sample was taken, is indicated above each track. The phenotype of each of these plants after incubation with X-glucuronide and removal of chlorophyll with ethanol is also indicated. Silenced lines are white (W), unsilenced lines blue (B). The non-transgenic control is designated (C). Sizes indicated on the filter were determined by migration of DNA oligonucleotides.

**Figure 2.** Silenced phenotypes in ihpRNA transformed plants and recombinase cloning into pHELLSGATE.

- Arabidopsis* seed samples from left to right: five independent CHS-ihpRNA lines; wild-type seed; *tt4*CHS mutant; three CHS-anti-sense lines and three CHS-co-suppression lines. The anti-sense and co-suppression lines were chosen as those showing the lightest seed-coat pigmentation.
- Arabidopsis* seed samples from four independent CHS-ihpRNA lines, chosen to reflect the range of seed-coat pigmentation, viewed under a light microscope.
- Four companion seed samples to (b) from left to right: *tt4*CHS mutant; the two anti-sense-silenced lines from Table 1; and wild-type seed.
- Three pots of *Arabidopsis* plant lines 25 days after germination. From left to right: wild-type; earliest-flowering anti-sense; and FLC1-pHANNIBAL-transformed line.
- Arabidopsis* transformed with EIN2-pHANNIBAL growing on 50 µM ACC. The larger, vigorous plantlet is an ethylene-insensitive EIN2-pHANNIBAL plant; the small plantlets are ethylene-sensitive wild-type plants.
- X-glucuronide-stained transgenic GUS tobacco plantlets segregating for presence (left) or absence (right) of the GUS-hpRNA transgene.
- Agarose gel of restriction enzyme-digested plasmid preparations from nine individual colonies recovered from *E. coli* transformed with a pHELLSGATE/400ntPCR-product recombination reaction. *Xho*I digestion (g) will release the 400 nt sense arm but not the ccdB fragment, and *Xba*I digestion (h) will release the 400 nt anti-sense arm but not the other ccdB fragment. Left-hand track in both gels contains size markers.

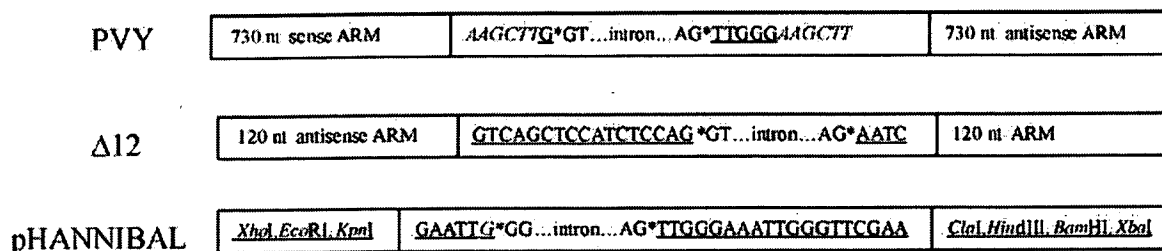


Figure 4. Splice junctions, loop regions and self-complementarity of hpRNA constructs: PVY,  $\Delta 12$ -desaturase, and pHANNIBAL.

Nucleotides underlined have no complement in the hpRNA and should form a loop structure. \*, splice point; *HindIII* site in PVY-hpRNA construct shown in italics.

PVY-Pro sequence) that should produce a hpRNA with a stem of 400 nt and 5' region of 300 unpaired nucleotides (Figure 1b). When 48 independent transformed plants, containing this construct, were challenged with PVY, 38 (=80%) of them were immune to the virus. This shows that an unpaired 5' extension of hpRNA does not abolish its ability to induce silencing, although its efficiency may be slightly reduced.

#### High-throughput vector

With the completion of the *Arabidopsis* genome project; the advent of micro-array technology; and the ever-increasing investigation into plant metabolic, perception and response pathways, a rapid, targeted way of silencing genes would be of major assistance. The high incidence and degree of silencing in plants transformed with pHANNIBAL constructs suggest that it could form the basis of a high-throughput silencing vector. However, one of the major obstacles in using pHANNIBAL for a large number of defined genes or a library of undefined genes would be cloning the hairpin arm sequences for each gene in the correct orientations.

Attempts to clone PCR products of sense and anti-sense arms, together with the appropriately cut pHANNIBAL vector as a single-step four-fragment ligation, failed to give efficient or reproducible results (data not shown). Therefore a construct (pHELLSGATE) was made (Figure 5) to take advantage of Gateway technology which facilitates easy cloning of PCR fragments (<http://www.invitrogen.com/content.cfm>). With this technology, a PCR fragment is generated (bordered with recognition sites attB1 and attB2) which is directionally recombined *in vitro* into a plasmid containing attP1 and attP2 sites using the commercially available recombinase preparation.

The pHELLSGATE vector was designed such that a single PCR product from primers with the appropriate attB1 and attB2 sites would be recombined into it simultaneously to form the two arms of the hairpin (Figure 5). The *ccdB* gene, which is lethal in standard *E. coli* strains such as DH5 $\alpha$  (but

not in DB3.1), was placed in the locations to be replaced by the arm sequences, ensuring that only recombinants containing both arms would be recovered. Placing a chloramphenicol-resistance gene within the intron gives a selection to ensure the retention of the intron in the recombinant plasmid. The pHELLSGATE vector was tested using 200 and 400 nt PCR products for two different genes. Many bacterial colonies were obtained on chloramphenicol-containing plates spread with DH5 $\alpha$  bacteria, transformed with the *in vitro* recombination reaction. Analysis of 24 colonies transformed with the 400 nt reaction and 36 colonies from the 200 nt reaction showed that, in both cases, all but one of the colonies contained the desired recombinant plasmid (Figure 2g,h). This was confirmed by sequence analysis (data not shown). These results show that this vector facilitates the rapid, efficient and simple production of hpRNA constructs. pHELLSGATE is a binary vector, with a high-copy-number origin of replication for ease of handling. Recombinant pHELLSGATE constructs can be directly transformed into *Agrobacterium* for transformation into plants. This system should lend itself to high-throughput applications.

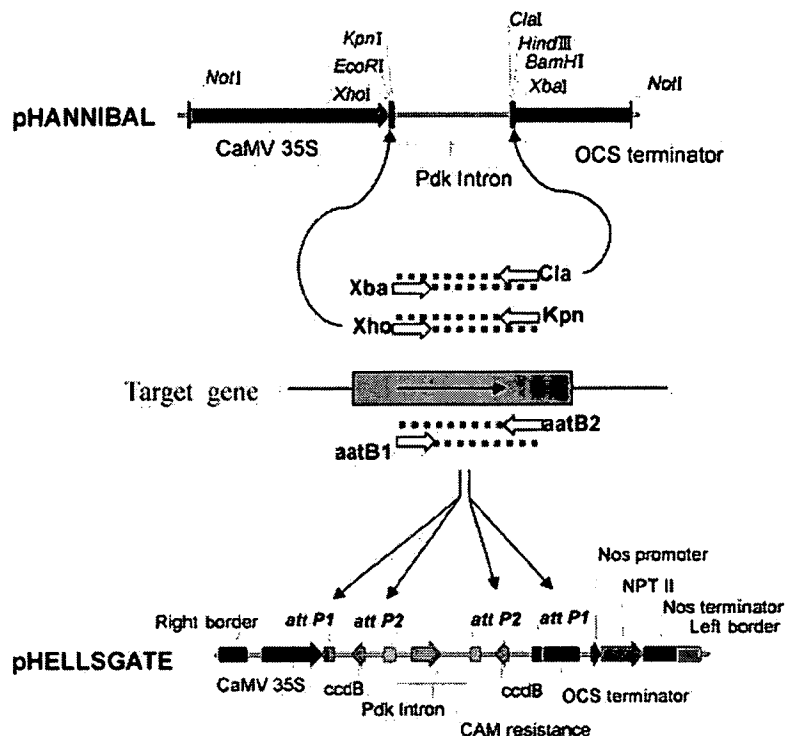
#### Discussion

Now that the genomes of a number of species have been completely sequenced, the challenge is to understand the functions and interplay of genes in an organism. The use of chemical mutagens, transposons and T-DNA tagging have been very useful in screening for mutants of individual genes. However, with these undirected methods it is often slow and laborious work to identify each mutant and to track down the gene responsible. RNAi has revolutionized the study of genes in *C. elegans* and *Drosophila*, with two groups recently reporting the systematic analysis of over 4000 genes on chromosomes I and III in *C. elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). By way of comparison, chromosome 2 of *Arabidopsis* has been entirely sequenced (Lin *et al.*, 2000) and the presence of 4037 genes has been predicted. Yet to undertake a systematic analysis



**Figure 5.** Maps and cloning strategies for pHANNIBAL and pHELLSGATE.

PCR products from the target gene are cloned into the polylinkers of pHANNIBAL conventionally; restriction sites added by the primers ensure the correct orientation of the resulting sense and anti-sense arms. The attB1 and attB2 sequences on a single PCR product facilitate the recombination of one sense-orientated and one anti-sense-orientated molecule into each molecule of pHELLSGATE when incubated with BP clonase. The complete sequences and annotations for pHANNIBAL and pHELLSGATE have been lodged at EMBL (Acc No: AJ311872 and AJ311874).



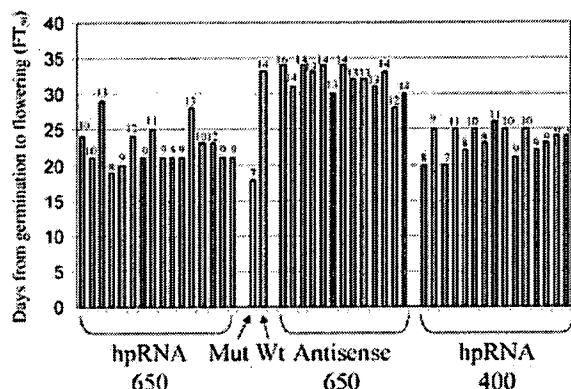
**Table 1.** Efficiency of hpRNA, co-suppression and anti-sense constructs at silencing a range of genes in a range of plant species

Gene	Species	Prom	Intron	Target	Arm (nt)	GenBank Acc No.	Co-ordinates	ihp RNA	hp RNA	Sense	Anti-sense
PPO	Tobacco	35S	Pdk	ORF	572	AX028815	172-844	21/30		5/54	
GUS	Tobacco	35S		ORF	800	S69414	1-800		23/48		
PVY	Tobacco	35S	Pdk	ORF	730	U09509	6278-7008	23/24	25/43	2/27	1/25
EIN2*	<i>Arabidopsis</i>	35S	Pdk	ORF	600	AF141202	538-1123	42/64			
FLC1*	<i>Arabidopsis</i>	35S	Pdk	ORF	650	AY034083	1-650	16/16			2/13
FLC1*	<i>Arabidopsis</i>	35S	Pdk	ORF	400	AY034083	250-650	15/15			
CHS	<i>Arabidopsis</i>	35S	Pdk	ORF	741	AF112086	248-1075	21/23		2/19	0/25
Δ12	<i>Arabidopsis</i>	Napin	Δ12a	3' UTR	120	L26296	1243-1363	30/30	44/63	4/41	3/21
Δ12	Cotton	Lectin		ORF	853	X97016	68-921		17/29		7/30
Δ12	Cotton	Δ12c	Δ12c	5' UTR	98	X97016	1-98	26/26			
Δ9	Cotton	Lectin		ORF	514	X95988	24-538		15/26		4/30
GUS	Rice	Ubi		ORF	560	S69414	1-560	12/14		3/10	1/8
Average percentage of silenced plants								90	58	13	12

The type of promoter (Prom), type of intron, length of arms and details of how to find the specific sequences of the arms for various gene-silencing constructs are shown. The last four columns show the number of primary independent transformants (or transformed lines where progeny were analysed) showing silencing/the number of transgenic plants produced from the primary transformation experiment. \*Silencing analysis was done on the progeny of the primary transformed plants.

of these genes using the conventional plant technologies of insertional mutagenesis would require vast resources. It has been calculated that to have a 90% chance of finding just one specific single gene (of about 1 kb) in *Arabidopsis* using T-DNA insertional mutagenesis would require the generation of about 350 000 independent transformants

(Krysan *et al.*, 1999). The work described in this paper facilitates a directed silencing which, when combined with the efficient, non-tissue culture transformation method for *Arabidopsis* (Clough and Bent, 1998), provides the tools that make the challenge of mirroring in plants, the gene discovery under way in nematodes, more feasible.



**Figure 6.** Flowering time in transgenic progeny from independent FLC1-pHANNIBAL and FLC1-anti-sense transformed C24 *Arabidopsis* lines. Flowering time (FT<sub>50</sub>) and leaf number (in figures above each column) for transgenic progeny from 16 and 15 independent plant lines transformed with pHANNIBAL constructs containing 650 and 400 nt arms of FLC1 sequence, respectively; 13 independent plants transformed with a conventional, 35S-driven, anti-sense construct containing the appropriate 650 nt of FLC1; the flc-13 mutant (white bar); and wild-type plants (cross-hatched bar). Wild-type plants were transgenic for GUS and kanamycin resistance, to allow the plants to be grown under identical conditions. The standard error for leaf numbers did not exceed 0.7 of 1 day for any plant line.

Using hpRNA constructs, we have obtained silenced plants for every gene that we targeted, irrespective of whether it was a viral gene, transgene or endogenous gene, and the silencing appears to be uniform within tissues in which the hpRNA is expressed. With ihpRNA constructs the efficiency averaged about 90%, and arms of 400–800 nt appear to be stable and effective. High levels of silencing were obtained with constructs having unmatched arm lengths, with arms as long as 853 nt or as little as 98 nt, and with arm sequences derived from coding, 3' or 5' untranslated regions of the target gene. These results suggest that ihpRNA constructs will be effective in a wide range of circumstances, and augur well for the generic use of the technology. The silencing was much more profound with ihpRNA constructs than either anti-sense or co-suppression constructs; some ihpRNA transformants were close to exhibiting a complete knock-out of the target endogenous gene. However, most of the ihpRNA plants showed dramatically reduced but detectable levels of target gene activity. This variation in degree of silencing in the ihpRNA plants may be a useful feature for gene discovery and genomics: complete silencing of genes required for basic cell function or development will probably be embryo-lethal and therefore not easily recovered using traditional tagging approaches, whereas the reduced gene expression caused by hpRNA constructs may give viable plants with phenotypes indicative of the role of the target gene.

Although the pHANNIBAL construct should be very useful for studying a modest number of genes (e.g. 10–

50), such as in a metabolic pathway, it would not be feasible with normal resources to use it for hundreds to thousands of genes. However, the pHELLSGATE vector has the potential to facilitate making large numbers of gene ihpRNA constructs rapidly and efficiently. The simple steps required, namely PCR, incubation of the PCR product with the vector and recombinase, selection of recombinant plasmid, and then transformation into *Agrobacterium*, are steps that could easily be automated. The templates for PCR could be the defined genes in an EST library using standard forward and reverse primers. Alternatively, given that ihpRNA constructs with arms as small as 98 nt give effective silencing, oligosynthesizers could be automated to systematically synthesize oligonucleotides of each computer-identified gene along a chromosome, or for genes for which no function is known, and pass these primers into an automated ihpRNA production system.

It has been shown that RNAi in *Drosophila* is directed by 21 nt dsRNA oligomers derived from the inducing dsRNA (Elbashir *et al.*, 2001; Zamore *et al.*, 2000). Similar 21–25 nt RNAs have also been found associated with PTGS in plants (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001). It is tempting to speculate that the minimum region of homology between an mRNA and the arms of an effective hpRNA will also be 21–25 nt. If so, this rule would allow the design of hpRNAs to silence a single member of a gene family, as such unique sequences are present in most gene families. Also, by choosing conserved regions, it may be possible to silence whole gene families using a single construct. However, these rules remain to be proven.

## Experimental procedures

### Plasmid construction

Standard gene cloning methods (Sambrook *et al.*, 1989) were used to make the gene constructs. The plasmids for dicot transformation were derived from pART7 and pART27 (Gleave, 1992), and those for monocot transformation were derived from pVec4 (Wang *et al.*, 1997; Wang *et al.*, 1998). The accession numbers of the gene sequences, and the co-ordinates of the sequences used in the hpRNA, co-suppression and anti-sense constructs, are shown in Table 1. The annotated sequences of pHANNIBAL, pKANNIBAL and pHELLSGATE are lodged with EMBL and have accession numbers AJ311872, AJ311873 and AJ311874, respectively. Constructs made in pHANNIBAL were subcloned as *NotI* fragments into pART27, then introduced into *Agrobacterium* strains AGL1 or LBA4404 either by electroporation or tri-parental mating. pHELLSGATE was maintained in *E. coli* strain DB3.1 (Invitrogen, Carlsbad, CA, USA) in which the *ccdB* gene is not lethal.

### Plant transformation

*Nicotiana tabacum* (W38), cotton and rice were transformed essentially as described by Ellis *et al.* (1987), Cousins *et al.* (1991) and Wang *et al.* (2001), respectively. *Arabidopsis* was transformed by the dipping method of Clough and Bent (1998).

### Analysis of transgenic plants

Northern blot analysis for the presence or absence of short RNAs was performed essentially as described by Wang *et al.* (2001).

Polyphenol oxidase (PPO) activity was measured using an oxygen electrode essentially as described by Robinson and Dry (1992). Rice and tobacco were tested for GUS activity using the histochemical stain X-glucuronide essentially as described by Jefferson *et al.* (1987). The reactions of plants to potato virus Y were analysed as described by Waterhouse *et al.* (1998). The activity of EIN2, which is required in the ethylene perception pathway, was observed by growing plants on media containing 1-aminocyclopropane-1-carboxylic acid (ACC) as described by Alonso *et al.* (1999). To identify lines silenced for EIN1, at least 30 progeny of each transformed line were germinated and grown on ACC-containing media.

To measure the effect of silencing FLC1, 20–30 seeds from each transgenic C24 *Arabidopsis* line, the transposon mutant flc13 (Sheldon *et al.*, 2000), and a control GUS line, were germinated and grown on kanamycin plates as described by Sheldon *et al.* (2000). The plants were scored daily over a 40-day period for the appearance of flowers. Flowering time (FT<sub>50</sub>) for each line was taken as the number of days after germination for 50% of the plants to show flowering. After flowering, the number of leaves of 10 randomly selected plants was counted for each line.

Chalcone synthase (CHS) activity was monitored by visual observation of stem and leaf colour in plants grown under high light, and by unaided or microscope-assisted visual observation of seed-coat colour. The seeds were collected after they had matured and dried on the plant. The relative flavonoid concentrations in seeds were determined by measuring extracts for absorbance between 490 and 530 nm in a Spectramax 340-PC (Molecular Devices Corporation, Sunnyvale, CA, USA). Duplicate extracts were made from 25 mg seed of each line, essentially as described by Gerats *et al.* (1982). The average absorbance value for each line was mathematically transformed to give relative values such that the *tt4* and wild-type seed became values of 0 and 100%, respectively.

The activity of  $\Delta 12$ - and  $\Delta 9$ -desaturase activity during lipid synthesis was estimated from the relative proportions of individual fatty acids in mature seed, as determined by routine methods for GC analysis of fatty acid methyl esters.

Unless otherwise stated, plants were considered to be showing silencing when they showed obvious appropriate phenotypic differences from wild-type plants, or when they had a gene activity that was reduced by at least 20%.

### AttB primers, PCR and recombination reaction for introduction of sequences into pHELLSGATE

Primers with attB1 and attB2 sequences were purchased from Life Technologies. Polymerase chain reactions (PCR) and *in vitro* BP clonase recombination reactions were carried out according to the manufacturer's instructions (Invitrogen). The recombination reaction product was either electroporated or heat-shocked into RbCl-treated DH5 $\alpha$  *E. coli*.

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# Exhibit 11

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## Double Jeopardy: Both Overexpression and Suppression of a Redox-Activated Plant Mitogen-Activated Protein Kinase Render Tobacco Plants Ozone Sensitive

Marcus A. Samuel and Brian E. Ellis<sup>1</sup>

Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, V6T 1Z3 Canada

In plants, the role of mitogen-activated protein kinase (MAPK) in reactive oxygen species (ROS)-based signal transduction processes is elusive. Despite the fact that ROS can induce MAPK activation, no direct genetic evidence has linked ROS-induced MAPK activation with the hypersensitive response, a form of programmed cell death. In tobacco, the major ROS-induced MAPK is salicylate-induced protein kinase (SIPK). We found through gain-of-function and loss-of-function approaches that both overexpression and RNA interference-based suppression of SIPK render the plant sensitive to ROS stress. Transgenic lines overexpressing a nonphosphorylatable version of SIPK were not ROS sensitive. Analysis of the MAPK activation profiles in ROS-stressed transgenic and wild-type plants revealed a striking interplay between SIPK and another MAPK (wound-induced protein kinase [WIPK]) in the different kinotypes. During continuous ozone exposure, abnormally prolonged activation of SIPK was seen in the SIPK-overexpression genotype, without WIPK activation, whereas strong and stable activation of WIPK was observed in the SIPK-suppressed lines. Thus, one role of activated SIPK in tobacco cells upon ROS stimulation appears to be control of the inactivation of WIPK.

### INTRODUCTION

Mitogen-activated protein kinase (MAPK) modules form a key part of the eukaryotic signal transduction network that links environmental inputs to a wide range of modifications of cellular functions, ranging from cell division to cell death. In plants, MAPK signaling has been implicated in defense against pathogens and herbivores, in cellular responses to auxin, abscisic acid, and other phytohormones, in cell cycle control, in the induction of programmed cell death, and in responses to abiotic stresses such as UV light and ozone (Zhang and Klessig, 1997; Kovtun et al., 1998; Romeis et al., 1999; Heimovaara-Dijkstra et al., 2000; Samuel et al., 2000; Nishihama et al., 2001; Yang et al., 2001; Miles et al., 2002).

A variety of stress responses have been found to involve the rapid activation of a specific subset of plant MAPKs, notably *Arabidopsis* MPK6 (Ichimura et al., 2000; Kovtun et al., 2000; Nühse et al., 2000; Yuasa et al., 2001) and its orthologs in other species, such as salicylic acid-induced protein kinase (SIPK) in tobacco (Zhang and Klessig, 1998a, 1998b; Romeis et al., 1999; Mikolajczyk et al., 2000; Samuel et al., 2000; Zhang et al., 2000) and salt stress-induced MAPK (SIMK) in alfalfa (Cardinale et al., 2000). Because many biotic

and abiotic stressors (virus infection, treatment with microbial elicitors, wounding, and osmotic stress) elicit a very rapid oxidative burst in plant cells, the apparent convergence of disparate stress signals on this particular MAPK node may be related to the sensitive response of MPK6/SIPK to redox perturbation.

Exposure to ozone immediately creates an oxidizing environment in plant tissues and triggers an array of cellular responses, including the accumulation of antioxidants, elicitation of pathogenesis-related proteins, deposition of phenols, induction of ethylene synthesis, suppression of primary metabolic activities such as photosynthesis, and eventually cell death (Darrall, 1989; Schraudner et al., 1992; Conklin and Last, 1995; Sharma and Davis, 1997; Tuomainen et al., 1997). Ozone enters the plant mesophyll through the stomata and diffuses through inner air spaces. In the cell wall and plasmalemma, it is converted spontaneously to reactive oxygen species (ROS) by contact with either water or membrane components (Sharma and Davis, 1997). The ozone-induced cell death process is influenced by the interaction of multiple signaling molecules, including salicylic acid, jasmonic acid, and ethylene (Orvar and Ellis, 1997; Overmyer et al., 2000; Rao et al., 2000).

One of the earliest responses elicited by ozone and other ROS generators in plants is the activation of specific MAPKs (Samuel et al., 2000; Desikan et al., 2001). The primary ROS-activated tobacco MAPK has been identified as the 46-kD

<sup>1</sup>To whom correspondence should be addressed. E-mail bee@interchange.ubc.ca; fax 604-822-2114.

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SIPK; a second MAPK, the 44-kD wound-induced protein kinase (WIPK), usually responds more weakly (Kumar and Klessig, 2000; Samuel et al., 2000).

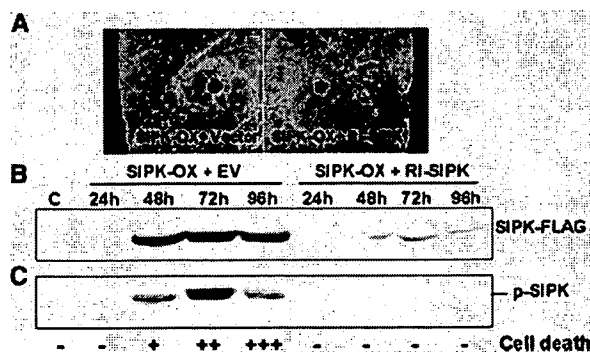
The rapid activation of these MAPKs suggests that their action on downstream targets could be important for the modulation of the cellular response to increased oxidative damage, but direct evidence for that role is lacking in plants. No intracellular substrates have been identified for either SIPK or WIPK, nor have loss-of-function genotypes been assessed for their ability to control redox stress. Stable overexpression or suppression of SIPK or WIPK in transgenic tobacco apparently did not result in the alteration of its activity (Yang et al., 2001). By contrast, transient overexpression of SIPK or its upstream activator, NtMEK2, in an active form has been shown to lead to the activation of either SIPK or both SIPK and WIPK, with associated induction of defense genes and hypersensitive response (HR)-like cell death (Yang et al., 2001; Zhang and Liu, 2001). This finding suggests that SIPK may play a role as a positive regulator in the cell death pathway.

The previously reported inability to produce SIPK-suppressed lines, and the lack of phenotype or alteration of SIPK activity reported for overexpression lines (Yang et al., 2001), have suggested that the normal functioning of this kinase may be essential for cell survival. However, we report here, using RNA interference (RNAi) technology, the recovery and analysis of transgenic tobacco plants in which SIPK is either overexpressed ectopically or largely eliminated. These plants display distinctive ozone response phenotypes that confirm the importance of SIPK activation for the effective control of ROS damage and also reveal an unexpected interplay between the activities of SIPK and WIPK.

## RESULTS

Infiltration of fully grown tobacco leaves with a suspension of *Agrobacterium tumefaciens* cells carrying a SIPK-FLAG overexpression construct resulted in the accumulation of the epitope-tagged SIPK protein in the infiltrated tissue within 48 h. In unstressed cells, endogenous SIPK was not phosphorylated at the TXY motif found in the activation loop of the kinase, as indicated by the absence of any signal in the control lane of a protein gel blot (Figure 1C) prepared using an anti-pMAPK antibody that specifically recognized the doubly phosphorylated protein. In the infiltrated tissue, however, at least a portion of the pool of SIPK became activated by 48 h after infiltration, with even greater activation observed by 72 h. In the same period, the infiltrated zones showed signs of tissue collapse, and by 96 h, these zones became completely necrotic (Figure 1A).

When leaves were coinfiltrated with *Agrobacterium* carrying the SIPK-FLAG overexpression construct plus an RNAi construct that targeted SIPK, both expression and activation of SIPK-FLAG were suppressed completely (Figures 1B and



**Figure 1.** Post-Transcriptional Gene Silencing-Induced Suppression of Cell Death Triggered by Transient Overexpression of SIPK.

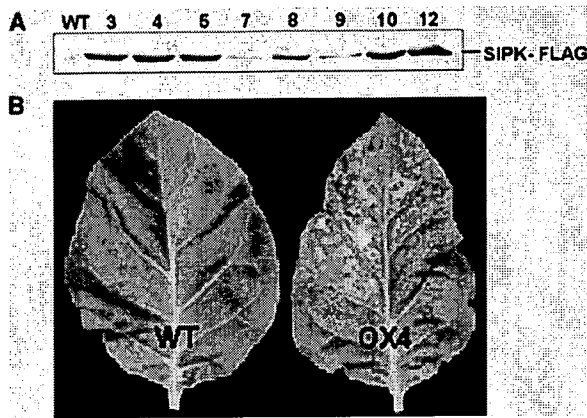
Coinfiltration with *Agrobacterium* containing the SIPK-FLAG construct along with the SIPK-RI construct inhibited the cell death process induced by transient overexpression of SIPK-FLAG alone (A). Protein samples extracted at different times after infiltration of the constructs were immunoblotted with either anti-FLAG antibody (B) or phospho-MAPK-specific antibody (anti-pERK) (C). -, +, ++, and +++ indicate the extent of visible lesions appearing in the infiltrated zones. EV, *agrobacterium* carrying empty vector.

1C). The cell death induced by the overexpression of SIPK-FLAG in the infiltrated zones also was eliminated (Figure 1A).

The cell death associated with the spontaneous activation of SIPK in overexpression (OX) transgenic cells suggested that it might be difficult to recover stably transformed lines using this construct, but cocultivation of tobacco leaf discs with the appropriate *Agrobacterium* culture and selection on kanamycin yielded a number of transgenic lines that were found to ectopically express a range of levels of SIPK-FLAG (Figure 2A). No spontaneous activation of SIPK was detected in these lines, all of which displayed normal growth and development phenotypes.

Transformation of tobacco leaf discs with the SIPK-RI construct also yielded stable transgenic lines, although with a sharply reduced frequency. In the recovered RI lines, silencing of endogenous SIPK expression was observed to varying degrees, ranging from partial reduction in both SIPK mRNA and protein to elimination of both products (Figures 3B and 3C). The specificity of this silencing was shown by the continued expression in most of the recovered RI lines of the closely related *NTF4* MAPK gene, whose cDNA sequence is 89% identical to that of *SIPK* (Figure 3D). The RI lines again showed largely normal growth and development phenotypes, although the most severely suppressed lines showed some modest tendency to dwarfing (data not shown).

Plants of both the OX and RI lines showed no signs of spontaneous cell death under normal growth conditions. However, exposure of mature OX or RI leaves to levels of ozone that caused no visible injury to wild-type plants (500 parts per billion [ppb]) resulted in the rapid appearance of small necrotic lesions on leaves of both the transgenic



**Figure 2.** Transgenic Tobacco Plants Overexpressing SIPK-FLAG Show Increased Ozone Sensitivity.

**(A)** Proteins (40  $\mu$ g) extracted from leaves of the different OX lines ectopically expressing SIPK-FLAG were immunoblotted using anti-FLAG antibody.

**(B)** Transgenic tobacco line OX4 and wild-type tobacco (Xanthi-nc) plants ( $n = 25$ ) were exposed to ozone (500 ppb) for 8 h. The treated leaves were photographed 24 h after exposure.

WT, wild type.

genotypes (Figures 2B and 3E). The kinetics of this oxidative stress damage were quite different. Lesions consistently appeared on the leaves of OX plants as early as 4 to 6 h, but visually similar lesions only appeared on RI leaves ~24 h later. In plants challenged with lower ozone concentrations (250 ppb), an analogous pattern was observed except that the necrotic responses were delayed until 48 h (OX) and 72 h (RI) (data not shown). When leaf discs prepared from the wild-type, OX, and RI genotypes were assayed for the loss of membrane integrity and associated ion leakage resulting from ozone exposure (500 ppb), differential timing of the damage response also was observed (Figure 4).

To assess *in situ* the relative levels of hydrogen peroxide accumulation induced by ozone exposure, control and ozone-treated leaf halves were infiltrated with 3,3'-diaminobenzidine solution. The staining patterns revealed no detectable levels of hydrogen peroxide in untreated leaves of any of the genotypes or in leaves of wild-type plants after 8 h of ozone exposure. However, strong 3,3'-diaminobenzidine staining was observed in both the OX and RI lines after ozone treatment (Figure 4B).

The observation that overexpression of SIPK-FLAG in infiltrated leaves was accompanied by the spontaneous activation of MAPK and by cell death raised the question of whether activation of the ectopically expressed protein was necessary for the induction of cell death. Therefore, site-directed mutagenesis was used to create a version of SIPK-FLAG in which the TEY motif found in the activation loop of SIPK had been converted to an AEF sequence. This modifi-

cation yielded a kinase that retained a low level of basal activity when the recombinant protein was assayed *in vitro* against myelin basic protein (Figure 5A), but it could not be activated further through dual phosphorylation of the activation loop by upstream MAPK kinases. Unlike the SIPK-FLAG construct, when transiently expressed in tobacco leaves, the SIPK(AEF)-FLAG construct failed to cause cell death in the infiltrated zone (data not shown).

Stably transformed tobacco plants expressing high levels of SIPK(AEF)-FLAG also were recovered readily after *Agrobacterium* cocultivation, and these plants displayed no visibly altered phenotype. Despite accumulating similar levels of the epitope-tagged kinase (Figure 5B), the ozone sensitivity of these SIPK(AEF) transgenic lines did not differ from that of wild-type plants (data not shown). This finding indicates that the heightened ozone sensitivity observed in SIPK-OX transgenic lines requires not only that the ectopically expressed kinase be expressed at high levels within the plant cell but that it have the capacity to become activated.

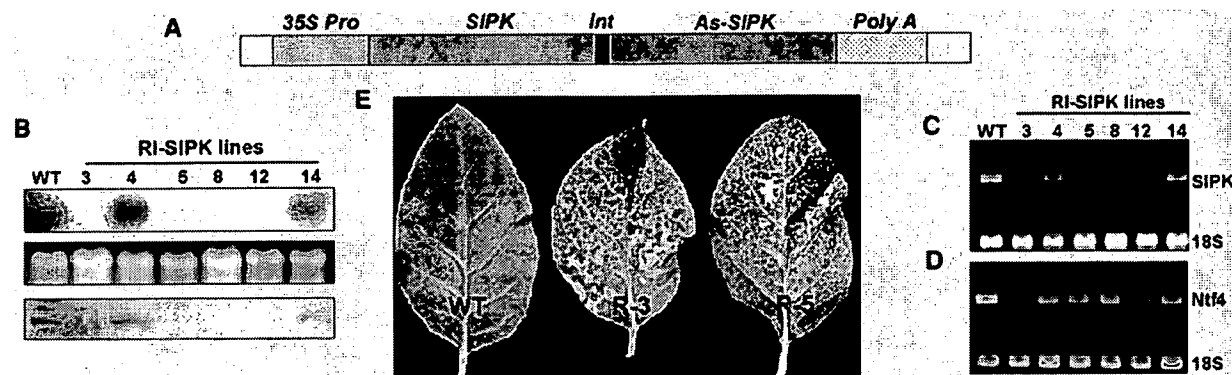
The activation status of both SIPK and WIPK in tobacco tissue extracts can be assessed either on protein gel blots using a phosphospecific antibody or by immunoprecipitation with antibodies that discriminate between SIPK and WIPK, followed by *in gel* or *in vitro* kinase activity assays. When the various transgenic and wild-type tobacco lines were monitored during a 30-min period of ozone exposure, striking differences in the pattern of kinase activation were observed among these genotypes (Figure 6).

As reported previously (Samuel et al., 2000), ozone treatment led to the rapid activation of SIPK in leaves of wild-type plants. This was accompanied by a much weaker activation of the smaller kinase, WIPK (Figure 6A). In the OX4 genotype, ozone exposure also led to SIPK activation, but the level of activation appeared to be depressed relative to the wild-type response, despite the presence of far greater amounts of ectopically expressed SIPK in the OX cells (Figures 6B and 6C). No activation of WIPK was detected in the OX tissue samples.

The SIPK(AEF) genotype presented a kinase activation profile that was very similar to that of the wild type. This indicates that flooding the cell with a nonactivatable version of SIPK (a potential dominant-negative form) does not interfere with the ability of the upstream MAPK cascade elements to transmit oxidant-induced signals to their cognate MAPKs.

Exposure of the RI genotype to ozone, on the other hand, yielded a very different MAPK activation profile. Very weak or no SIPK activation was detected, as would be predicted for a genotype in which SIPK expression has been suppressed by post-transcriptional gene silencing (Figures 6A and 6C). Instead, ozone exposure produced strong and specific activation of WIPK. The identity of these highly activated kinases in ozone-treated leaves of each genotype was confirmed through immunoprecipitation of the 30-min ozone-treated protein extracts with either SIPK- or WIPK-specific antibodies, followed by *in gel* kinase assays (Figures 6D and 6E).

Aside from the unexpected massive activation of WIPK, the stability of that activation also was strikingly different in



**Figure 3.** SIPK-Suppressed Lines Also Are Sensitive to Ozone.

(A) RNAi construct under the control of the 35S promoter of *Cauliflower mosaic virus*.

(B) SIPK is suppressed in four of the six PCR-positive lines. RNA gel blot analysis was performed using total RNA (15  $\mu$ g) extracted from wild-type and SIPK-RI lines and probed with the radiolabeled C-terminal fragment of the *SIPK* ORF. Autoradiography revealed essentially no SIPK mRNA in four of the six PCR-positive lines (top). Ethidium bromide staining of the gel showed equal loading of RNA (middle). Immunoblot analysis of protein samples from the same lines indicated the absence of detectable amounts of SIPK protein in all four SIPK-suppressed lines (bottom).

(C) Similar results were observed when reverse transcriptase-mediated PCR was conducted using *SIPK*-specific primers.

(D) *NtF4* gene expression in the SIPK-suppressed lines was analyzed by reverse transcriptase-mediated PCR using gene-specific primers.

(E) SIPK-suppressed lines R3 and R5 display ozone-sensitive phenotypes. Plants ( $n = 15$ ) of SIPK-suppressed tobacco transgenic lines R3 and R5, together with wild-type plants, were exposed to ozone (500 ppb) for 8 h per day for 2 days. The treated leaves were photographed 24 h after the end of 2 days of exposure.

WT, wild type.

this genetic background. Normally, when oxidants trigger a rapid activation of SIPK, it is a transient response. The activation is effectively lost within 1 h, even under conditions of continuous oxidant stimulus, as seen in Figure 7A (wild-type lane). However, in the RI genotype, WIPK was not only activated rapidly but the pool of this MAPK remained continuously active for up to 8 h after the initiation of the response (Figure 7A, RI lane). Although normally there is far less WIPK than SIPK present in tobacco leaves (Zhang and Klessig, 1998b), the high activation signal observed in the RI tissue extracts did not appear to reflect increased levels of WIPK protein in this genotype compared with wild-type plants, as assessed by protein gel blot analysis (Figure 7C).

Interestingly, kinase activation by ozone in the OX genotype also was prolonged abnormally, relative to that seen in ozone-treated wild-type plants, but in this case, the active kinase was SIPK rather than WIPK (Figure 7B). In addition, unlike the hyperactivated WIPK pool, the extended activation of SIPK in the OX line was more transient and disappeared within 4 h. This is approximately the time at which visible lesions began appearing on ozone-treated OX leaves.

Examination of the temporal response of the two genes (*GST* [glutathione S-transferase] and *cAPX* [cytosolic ascorbate peroxidase]) whose expression was induced strongly by ozone treatment revealed that the loss of SIPK signaling in the RI genotype resulted in a delayed response in the expression of both genes. In the OX line, the prolonged activa-

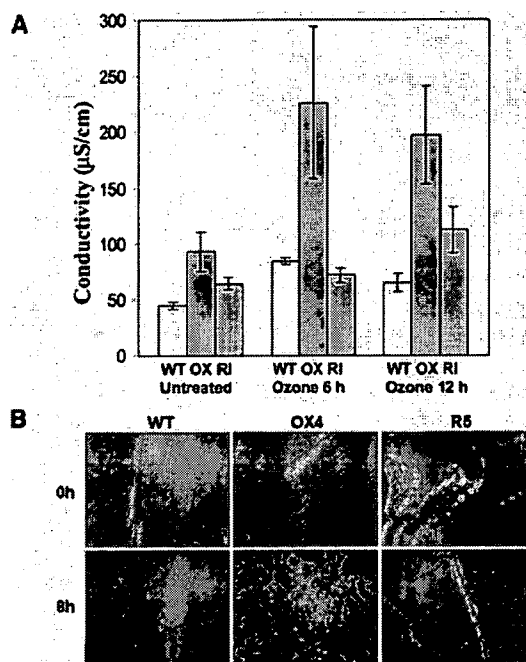
tion of SIPK signaling resulted in the suppression of *GST* induction, whereas *APX* gene expression was unaffected (Figures 8A and 8B).

## DISCUSSION

Plant cells must deal constantly with ROS from a range of sources, including photooxidation, mitochondrial electron transport, flavin oxidase by-products, and environmental insults such as UV light, ozone, and ionizing radiation. Against this background, ROS pulses ("oxidative bursts") also can occur within cells, usually as very early responses to localized challenges to cellular integrity such as wounding and pathogen assault. These pulses may serve in multiple functions, including activation of redox protection mechanisms, modulation of intracellular signal transduction pathways, and transmission of systemic signals to neighboring cells.

A severe oxidative challenge that overwhelms local protective measures ultimately will lead to cell death. The archetype for this outcome is the HR response induced during incompatible host-pathogen interactions. Similar lesions are induced by exposure to increased levels of ozone or UV light. The exact process by which cellular integrity fails is unclear, but the notion that HR represents a form of genetically programmed cell death is supported by the identification of nu-





**Figure 4.** Quantitation of Ozone-Induced Cell Death and Hydrogen Peroxide Accumulation in SIPK Kinotypes.

(A) Ion leakage from leaf discs (five each) of the third and fourth leaves of wild-type, OX4, and RI5 lines was assessed as an indicator of the loss of membrane integrity 6 and 12 h after the initiation of ozone exposure (500 ppb). The data presented are means and standard deviations from three independent experiments.

(B) 3,3'-Diaminobenzidine staining to detect hydrogen peroxide accumulation in ozone-treated leaves of SIPK kinotypes.

WT, wild type.

merous mutants affected in the process of lesion formation (Richberg et al., 1998).

The correlation of ROS pulses with the cell death process has been described extensively. Treatments such as chilling, wounding, pathogen infection, UV irradiation, and ozone exposure rapidly induce ROS accumulation in plant cells, followed later by lesion development. However, despite these correlative observations, a functional link between ROS accumulation and local lesion formation has yet to be defined.

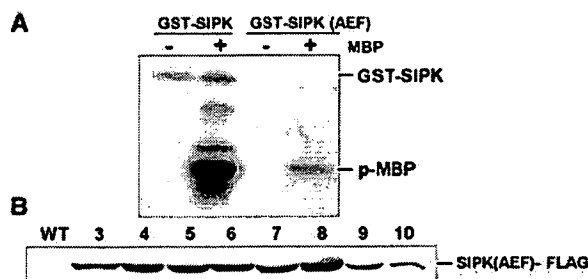
It is striking that so many stresses that elicit ROS accumulation in plant cells consistently appear to activate MAPK modules as one of their earliest effects (Seo et al., 1995; Zhang and Klessig, 1998b; Allan et al., 2001; Desikan et al., 2001; Orozco-Cardenas et al., 2001). The MAPK observed most consistently to be activated by both applied stresses and ROS is SIPK in tobacco (Samuel et al., 2000; Miles et al., 2002) or its apparent orthologs in other species, such as MPK6 in Arabidopsis (Kovtun et al., 2000; Yuasa et al., 2001) and SIMK in alfalfa (Cardinale et al., 2000). This pattern suggests that

SIPK activation might play an important role in determining the response and ultimate fate of the stressed cells.

Links between ROS-associated cell death and MAPK signaling have been reported for a number of nonplant systems. Hydrogen peroxide-induced cell death in cultured mammalian oligodendrocyte cells is inhibited by PD98059, a specific inhibitor of MEK, the upstream kinase of the ERK1/2 MAPK (Bhat and Zhang, 1999), whereas delayed and prolonged activation of p44 and p42 MAPKs is critical for genistein-induced programmed cell death in rat primary cortical neurons (Linford et al., 2001). Similarly, delayed and persistent activation of ERK1/2 is associated with glutamate-induced oxidative cytotoxicity in neuronal cell lines (Stanciu et al., 2000).

There also is evidence that ROS-activated MAPKs may play analogous roles in plant cells. Cell death induced in Arabidopsis cell suspension cultures by treatment with a bacterial elicitor (harpin) is inhibited when the cells are treated with the MEK inhibitor PD98059 (Desikan et al., 1999), whereas pretreatment of tobacco cells with staurosporine, a general protein kinase inhibitor, suppresses the cell death normally induced by exposure to fungal elicitors (Suzuki et al., 1999).

Genetic manipulation experiments also have implicated MAPK activation in the cell death process. In Arabidopsis plants overexpressing constitutively active forms of the MAPK kinases AtMEK4 and AtMEK5 under the control of an inducible promoter, HR-like lesions appeared after induction with dexamethasone, and lesion formation was preceded by the activation of endogenous MAPKs and the accumulation of hydrogen peroxide (Ren et al., 2002). Transient overexpression of a constitutively active form of a MAPK kinase (NtMEK2) in tobacco also led to the sustained activation of MAPKs, identified

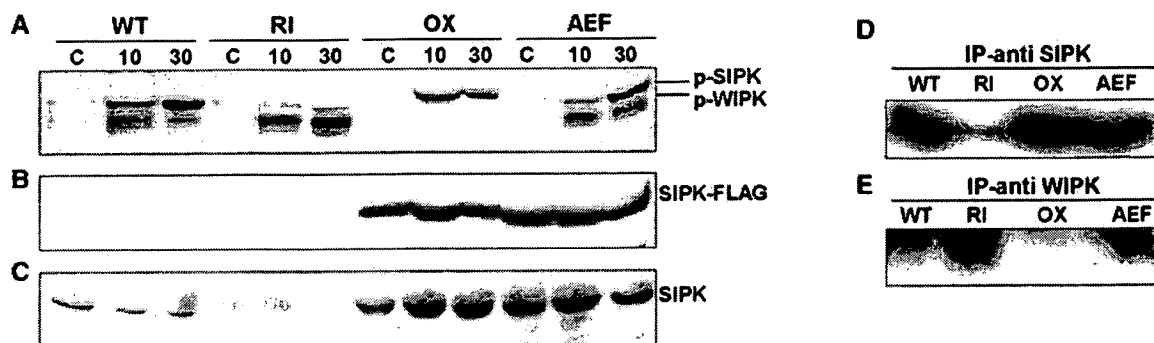


**Figure 5.** Activity and Expression of Mutagenized SIPK.

(A) The recombinant SIPK activation loop mutant is less active than wild-type SIPK. Myelin basic protein (MBP)-phosphorylating activities of SIPK and SIPK(AEF) were measured by incubating recombinant proteins (5 µg) with 5 µg of MBP, as described in Methods. The phospho-MBP product was visualized through autoradiography after SDS-PAGE fractionation.

(B) SIPK(AEF) transgenic lines show high expression of the transgene product. Proteins (40 µg) extracted from the different lines overexpressing SIPK(AEF)-FLAG were fractionated by SDS-PAGE and immunoblotted using anti-FLAG antibody.

WT, wild type.



**Figure 6.** Differential Ozone-Induced Activation of SIPK and WIPK in SIPK Kinotypes.

(A) Crude protein extracts prepared from ozone-exposed tissues from T1 lines of the different SIPK kinotypes (RI5, OX4, and AEF8) and the wild type were resolved on a 10% polyacrylamide gel, blotted, and probed with an anti-phospho-ERK antibody to recognize phospho-MAPK forms. (B) The same blot was probed subsequently using an anti-FLAG antibody to detect ectopic expression of the transgene product in the different kinotypes.

(C) A replicate protein gel blot was analyzed using a SIPK-specific antibody, revealing high expression of SIPK forms in the overexpressor lines and its absence in the SIPK-suppressed lines.

(D) and (E) Protein samples prepared from ozone-exposed (30 min) tissues from the different kinotypes were immunoprecipitated with either SIPK-specific (D) or WIPK-specific (E) antibodies. The immunoprecipitates were subjected to an in gel kinase assay, as described in Methods. WT, wild type.

as SIPK and WIPK, and to the death of the infiltrated tissue (Yang et al., 2001). Transient overexpression of SIPK itself was shown subsequently to result in the formation of HR-like lesions, but only in young leaves (Zhang and Liu, 2001).

We have confirmed that ectopic SIPK overexpression leads to the appearance of high levels of the activated kinase in *Agrobacterium*-infiltrated tobacco tissue and to rapid cell death (Figure 1). On the other hand, when stably transformed tobacco plants were produced that overexpressed epitope-tagged SIPK (Figure 2), they displayed no visible phenotype. When exposed to ozone, however, the transgenic SIPK-OX plants proved to be much more sensitive than the nontransgenic parental line, indicating that ROS-induced cell death was controlled less effectively in the overexpression genotype.

Although this pattern is consistent with the results of NtMEK2 or SIPK-OX transient expression, its physiological relevance remains uncertain, because we know little about the effects of the accumulation of nonphysiological levels of active signal components on cellular function. To unambiguously identify a functional relationship between ROS activation of SIPK and ROS-induced cell death, we turned to the creation of defined loss-of-function mutants.

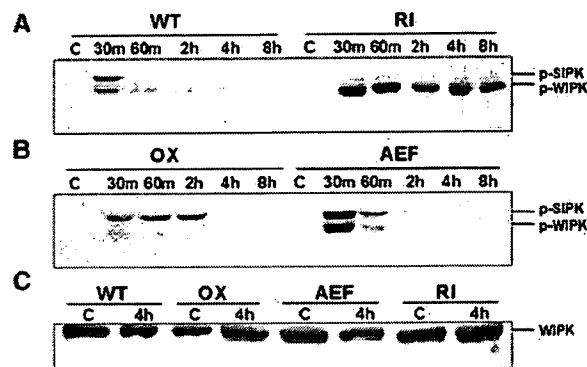
The modification of SIPK function in transgenic tobacco plants using either conventional gene-silencing methods (co-suppression and antisense-mediated suppression) or overexpression of dominant-negative forms proved ineffective (Yang et al., 2001) (data not shown). However, expression of an intron-containing "hairpin RNA" (Smith et al., 2000) designed to target a unique tract within the SIPK coding sequence yielded a number of transgenic plants in which SIPK expression was sup-

pressed severely and specifically through post-transcriptional gene silencing. Loss of SIPK had no obvious phenotypic consequences for plants grown under normal greenhouse conditions.

Given the sensitivity of SIPK-OX lines to ozone, it might have been predicted that the absence of this kinase would have no effects, or perhaps even positive effects, on the ozone sensitivity of the SIPK-RI lines. Instead, after ozone treatments that induced no visible damage on wild-type plants, the SIPK-RI lines developed numerous lesions on their middle leaves within 24 h. Thus, the inability of the suppressed genotype to generate and activate SIPK compromises the cell's ability to manage ROS stress and to control cell death, although apparently on a different time scale from that observed in SIPK-OX plants.

Which facet of ROS-stress management has been compromised in SIPK-OX and SIPK-RI plants is not clear. No constitutive hydrogen peroxide accumulation was detected in any of the genotypes, suggesting that their heightened ozone sensitivity is not the consequence of a preexisting accumulation of ROS. Instead, it appears that alteration of the normal ozone-induced MAPK activation process, through either unregulated overexpression or suppression, creates an inability to cope with increased redox stress. Examination of the transcriptional activity of two genes whose mRNAs accumulate rapidly after ozone exposure showed that the response of both genes was affected differently (Figure 8).

Expression of *cAPX*, which encodes a major ROS-scavenging enzyme, was induced less effectively by ozone in RI plants, whereas it was unaffected in the OX line. Antisense suppression of *cAPX* was shown previously to create hypersensitivity to both ozone (Orvar and Ellis, 1997) and pathogens (Mittler



**Figure 7.** Loss of SIPK Has Differential Effects on the Expression of Ozone-Induced Genes and Leads to the Hyperactivation of WIPK.

(A) and (B) Extended ozone exposure reveals strong and prolonged activation of WIPK in the RI line. A temporal profile of the phosphorylation status of SIPK and WIPK was generated through anti-p-ERK immunoblotting of crude proteins extracted from tissues of either wild-type and RI lines (A) or OX and AEF lines (B) exposed to ozone for different times (0 to 8 h).

(C) Alteration of SIPK does not lead to changes in the amount of WIPK. Protein extracts from untreated and 4-h ozone-treated tissues from different kinotypes were immunoblotted with anti-WIPK antibody. WT, wild type.

et al., 1999) in transgenic tobacco plants. On the other hand, ozone-induced expression of *GST*, a general cellular protectant, was suppressed strongly in the OX line, but its expression was delayed markedly in the RI line. In Arabidopsis, both hydrogen peroxide and ozone induce *GST* expression (Clayton et al., 1999; Grant et al., 2000), and this expression has been demonstrated to require the activity of an unidentified 48-kD MAPK and calcium ion influx. Calcium channel activity also is essential for the ROS activation of SIPK in tobacco (Samuel et al., 2000).

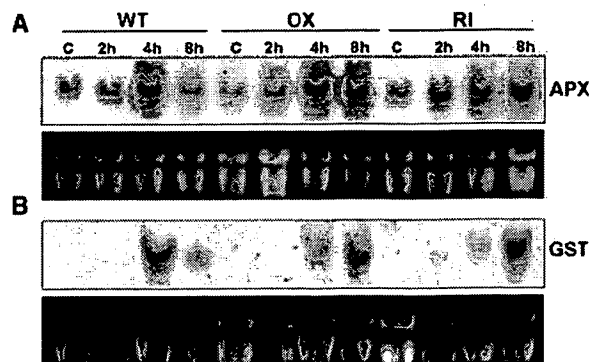
The delayed response of the antioxidant genes in the RI line could result in increased early accumulation of ROS (Figure 4B), which could lead to a necrotic cell death process. In the OX line, although the *cAPX* gene response to ozone appeared to be normal, the antioxidant response clearly was unable to contain the increasing ROS levels associated with extended SIPK activation (Figure 4B). MAPK activation has been linked previously to increased ROS accumulation in Arabidopsis (Ren et al., 2002). A broader comparison of transcript profiles should generate useful insights into other connections between the transmission of redox signals by SIPK and the ability of the cell to avoid oxidative cell death.

Another aspect of the link between SIPK activation and cell death is revealed in the pattern of MAPK activation in ROS-stressed plants. The activation of SIPK by ozone occurred within 10 min in SIPK-OX plants but was not reversed for 4 h, by which time cell death already was becoming visible. This outcome is similar to the association of the prolonged activa-

tion of mammalian ERK with the induction of programmed cell death in neurons (Stanciu et al., 2000). Considered together with the results of the transient expression experiments, this finding demonstrates that the unregulated continuous activity of SIPK within plant cells profoundly affects normal homeostatic mechanisms.

The absence of SIPK in the SIPK-RI genotype also led to premature cell death under redox stress conditions, but in this case, the hyperactivated species observed was WIPK rather than SIPK. There have been other indications that WIPK plays a central role in plant stress signaling. This gene was identified originally on the basis of its rapid and transient induction upon wounding of tobacco leaves (Seo et al., 1995), and the gene product was shown later to be activated transiently by wounding (Seo et al., 1999) and by various other stresses (Romeis et al., 1999; Zhang et al., 2000). WIPK activation usually is accompanied by the activation of SIPK, but SIPK and WIPK do not always respond in unison; some oxidative stresses appear to activate SIPK preferentially and leave WIPK unaffected (Kumar and Klessig, 2000; Samuel et al., 2000).

WIPK activity, either alone or together with SIPK, has been suggested to be involved in the induction of cell death in cultured tobacco cells by specific fungal elicitor treatments (Zhang et al., 2000). Pretreatment of the elicited cells with staurosporine and K252A (protein kinase inhibitors) completely suppressed both WIPK activation and cell death. However, transient overexpression of WIPK did not result in its activation and failed to induce cell death in infiltrated tobacco leaves, unlike overexpression of SIPK (Zhang and Liu, 2001). In another study, the stable overexpression of WIPK in transgenic tobacco was accompanied by the constitutive expression of protease inhibitor II and the accumulation of methyl jasmonate



**Figure 8.** Alteration of SIPK Signaling Affects the Expression of *GST* and *cAPX*.

RNA gel blot analysis of the accumulation of *cAPX* (A) and *GST* mRNA (B) in wild-type, SIPK-overexpressing, and SIPK-suppressed transgenic tobacco. Plants were exposed to ambient air (C) or 500 ppb of ozone for 2, 4, and 8 h, and total RNA was harvested from the third and fourth leaves.

WT, wild type.

(Seo et al., 1999), but the oxidative stress sensitivity of the WIPK-OX lines was not reported.

How SIPK elimination leads to the prolonged hyperactivation of WIPK is unknown, but various possibilities suggest themselves. If NtMEK2 is the sole upstream MAPK kinase responsible for the activation of both SIPK and WIPK, these two MAPKs may normally compete for binding to NtMEK2. However, basal levels of SIPK in unstimulated tobacco cells are much higher (10-fold) than those of WIPK (Zhang and Klessig, 1998b). In the absence of competition from SIPK, activation of WIPK by NtMEK2 activation in SIPK-RI cells may be much more efficient than usual. This scenario also might explain why WIPK remains largely inactivated in ozone-treated SIPK-OX tissues in which an excess of SIPK is present. However, although this model accounts for WIPK hyperactivation, it does not necessarily explain why that activation is prolonged abnormally.

Alternatively, one of the normal roles of activated SIPK may be the direct or indirect regulation of WIPK activity. Both dual-specificity phosphoprotein phosphatases (MKP) and Ser/Thr phosphatases have been implicated in inactivating MAPK pathways in mammalian and plant models (Brondello et al., 1997; Meskiene et al., 1998; Ulm et al., 2001; Westermarck et al., 2001). If SIPK activity is required for the induction or activation of a protein phosphatase that normally acts upon phospho-WIPK, the absence of SIPK from oxidant-stressed SIPK-RI cells would create a situation in which WIPK could be activated by its cognate MAPK kinase but could not be inactivated subsequently.

In this regard, it is interesting that Arabidopsis plants in which a dual-specificity phosphatase (AtMKP-1) has been mutated by T-DNA insertional mutagenesis display increased activation of an unidentified ~49-kD MAPK and are more susceptible to ROS-generating stresses (e.g., UV light) (Ulm et al., 2001). On the other hand, MP2C, an alfalfa Ser/Thr phosphatase belonging to the PP2C class, has been shown to be a negative regulator of the MAPK pathway involving stress-activated MAPK, an apparent ortholog of WIPK (Meskiene et al., 1998). Both classes of protein phosphatase could be involved in cross-regulation mechanisms. Resolution of this question, and of the relative importance of the loss of SIPK activity versus the enhancement of WIPK activity in controlling oxidant-induced cell death, will require the development and analysis of other relevant single and multiple loss-of-function genotypes. These studies are now under way.

## METHODS

### Plant Material and Treatment

Tobacco (*Nicotiana tabacum*) plants of all genotypes were grown for 6 weeks in soil under controlled environmental conditions (25/20°C, 16-h-light/8-h-dark cycle) and then exposed to ozone (500 parts per billion) and harvested as described previously (Orvar and Ellis, 1997).

### Recombinant Protein Production

The open reading frame (ORF) of salicylate-induced protein kinase (SIPK) was amplified by reverse transcriptase-mediated (RT) PCR using gene-specific primers and RNA isolated from untreated leaves of tobacco cv Xanthi-nc. The amplicon was cloned in frame into the expression vector pGEX 4T-3. Mutations in the activation loop of SIPK were introduced using a PCR-mediated approach, taking advantage of the unique NheI restriction site close to the activation loop. The mutational primers were designed so that the mutant form would code for AEF instead of TEY at amino acid positions Thr-218 and Tyr-220.

The SIPK(AEF) gene construct then was cloned into pGEX 4T-3. The recombinant glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 cells by induction with 0.1 mM isopropylthio- $\beta$ -galactoside for 4 h at 25°C, followed by purification according to the manufacturer's protocol (Amersham Pharmacia). The different constructs were sequenced to confirm the changes and the absence of mismatches.

### Intron-Spliced Hairpin Loop RNA-SIPK Construct

The double-stranded RNA interference construct was tailored through a PCR-mediated approach using the N-terminal sequence of the SIPK ORF. A minimal intron based on the splice junctions and flanking regions of the fourth intron of *AtIMPK6* (the Arabidopsis ortholog of SIPK) was incorporated into the sense-strand primer. The sense strand then was amplified using a primer combination that generated an EcoRI cleavage site and intron-XbaI sequence on the opposite ends of the product, whereas the antisense strand was amplified using a primer combination that added BamHI and XbaI sites on the opposite ends of the product. These two products were directionally cloned into EcoRI-BamHI-processed Bin19/pRT101 through a triple ligation, which placed the RNA interference construct under the control of the 35S promoter of *Cauliflower mosaic virus* (Figure 3A).

### Binary Vector Construction and Plant Transformation

The different SIPK overexpression constructs were tagged with a C-terminal FLAG epitope through a PCR-mediated approach, followed by ligation into the plant expression vector Bin19/pRT101, which contains an *nptII*-selectable marker. All of the constructs were sequenced to confirm the presence of appropriate changes. The recombinant binary vector was used to transform competent *Agrobacterium tumefaciens* (EHA105) cells by a freeze-thaw transformation procedure.

Agrobacterium-mediated transformation of tobacco (cv Xanthi-nc) was performed using a leaf disc cocultivation procedure. Transformants were selected on half-strength Murashige and Skoog (1962) culture medium containing 50 mg/L kanamycin. Surviving plantlets were screened by PCR using 35S forward and gene-specific reverse primer combinations. Positive transformants then were screened by protein gel blot analysis (see below) using an anti-FLAG antibody for the SIPK overexpression lines and anti-SIPK antibodies to assess the RI suppression lines.

The confirmed transgenic lines were transferred to soil and grown to maturity, and seeds were collected. The T1 seeds were germinated on half-strength Murashige and Skoog (1962) medium with 50 mg/L kanamycin, and antibiotic-resistant plants were transferred to soil and grown under controlled conditions.

### Transient Transformation Using *Agrobacterium* Infiltration

Four- to 6-week-old wild-type tobacco plants (cv Xanthi-nc) were used for infiltration experiments as described previously (Yang et al., 2001). This involved leaf infiltration with a mixed culture (OD of 0.4 at 600 nm) of *Agrobacterium* EHA105 containing the SIPK-FLAG overexpression construct plus an equal population of *Agrobacterium* containing either the empty vector or the SIPK-Rl construct. At the indicated times (Figure 1), the infiltrated area was cut from the leaf, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until further analysis.

### RNA Gel Blot and RT-PCR Analysis

Total RNA (15  $\mu\text{g}$ ) was resolved on 1% agarose-formaldehyde gels, blotted, and probed as described previously (Orvar and Ellis, 1997). The 600-bp C-terminal fragment of the SIPK ORF and PCR-amplified fragments of the cytosolic ascorbate peroxidase (*cAPX*) and *GST* were used as probes. Gene-specific primers were used to amplify the ORFs of *cAPX* (forward, 5'-AGAACAATTGCTATGGGTAAGTG-3'; reverse, 5'-GCAAGCTTAAGCTTCAGCAAAT-3') and *GST* (forward, 5'-ATGGCGATCAAAGTCCATGGTA-3'; reverse, 5'-TTTTTGCAGCTTCTCCAATCCC-3') using cDNA as the template.

The cDNA was synthesized from total RNA extracted from control and ozone-exposed tissues of the different genotypes/treatments using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). RT-PCR was performed using gene-specific primers designed to target either *SIPK* (25 cycles) or *NTF4* (30 cycles). The number of cycles was adjusted so that the amplification was within the linear range. As an internal control, 18S ribosomal cDNA was amplified using a 1:4 ratio of 18S-specific primers to competitor's DNA fragments provided by Ambion (Austin, TX).

### Protein Extraction and Protein Gel Blot Analysis

Total protein extracts were prepared (40 to 80  $\mu\text{g}$ ) and used for protein gel blot analysis as described previously (Samuel et al., 2000). A primary antibody dilution of 1:1000 was used for anti-pERK (New England Biolabs, Beverly, MA), and a dilution of 1:5000 was used for anti-SIPK, anti-wound-induced protein kinase (WIPK) (Seo et al., 1999; Y. Ohashi, personal communication), and anti-FLAG (Sigma) antibodies.

### Immune Complex Kinase Assay

Immunoprecipitations were performed as described previously (Samuel et al., 2000) using 250  $\mu\text{g}$  of extracted protein together with 5  $\mu\text{g}$  of either anti-SIPK or anti-WIPK antibodies. The immunoprecipitates were analyzed in an in gel kinase assay as described previously using myelin basic protein as the substrate (Zhang and Klessig, 1997).

### In Vitro Kinase Assays

GST fusion proteins (5  $\mu\text{g}$ ) of the wild-type and mutant SIPK(AEF) were incubated with 5  $\mu\text{g}$  of myelin basic protein and 10  $\mu\text{Ci}$  of  $\gamma\text{-}^{32}\text{P}$ -labeled ATP ( $>5000\text{ Ci/mmol}$ ) (Amersham Pharmacia) in a 20- $\mu\text{L}$  reaction mixture (20 mM Hepes, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 5 mM  $\beta$ -mercaptoethanol, 2 mM  $\text{Na}_2\text{VO}_4$ , and 20 mM  $\beta$ -glycerophos-

phate) at  $30^{\circ}\text{C}$  for 30 min. The reaction was stopped with 6  $\times$  SDS loading buffer, and the samples were resolved on a 15% polyacrylamide gel, blotted onto a nylon membrane, and visualized by autoradiography.

### Ion-Leakage Assay

Five leaf discs (9 mm) were cut from each of the third and fourth leaves of ozone-exposed and untreated plants of the wild type, OX4, and R15 lines. The 10 leaf discs were incubated in 5 mL of deionized water at  $25^{\circ}\text{C}$  on a gyratory shaker at 110 rpm for 4 h, and the conductivity of the solution was measured as described previously (Mittler et al., 1999).

### In Situ Staining for Hydrogen Peroxide

Hydrogen peroxide was visualized in situ by 3,3'-diaminobenzidine staining performed essentially according to Torres et al. (2002). Leaf halves were collected after 8 h of ozone exposure (500 parts per billion) and vacuum infiltrated with the 3,3'-diaminobenzidine (1 mg/mL) solution. Infiltrated leaves were placed under high humidity until brown precipitation was observed (5 to 6 h) and then fixed with a solution of ethanol:lactic acid:glycerol (3:1:1, v/v) for 2 days, followed by further clearing in methanol. Unless indicated otherwise, all experiments were repeated with consistent results.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

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# Exhibit 12

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## A Classical Arabinogalactan Protein Is Essential for the Initiation of Female Gametogenesis in Arabidopsis

Gerardo Acosta-García and Jean-Philippe Vielle-Calzada<sup>1</sup>

Laboratory of Reproductive Development and Apomixis, Department of Genetic Engineering, Centro de Investigación y Estudios Avanzados, Unidad Irapuato, Irapuato Guanajuato, Mexico

Classical arabinogalactan proteins (AGPs) are an abundant class of cell surface proteoglycans widely distributed in flowering plants. We have used a combination of enhancer detection tagging and RNA interference (RNAi)-induced posttranscriptional silencing to demonstrate that *AGP18*, a gene encoding a classical arabinogalactan protein, is essential for female gametogenesis in *Arabidopsis thaliana*. *AGP18* is expressed in cells that spatially and temporally define the sporophytic to gametophytic transition and during early stages of seed development. More than 75% of the T1 transformants resulted in T2 lines showing reduced seed set during at least three consecutive generations but no additional developmental defects. *AGP18*-silenced T2 lines showed reduced *AGP18* transcript levels in female reproductive organs, the presence of 21-bp RNA fragments specific to the *AGP18* gene, and the absence of in situ *AGP18* mRNA localization in developing ovules. Reciprocal crosses to wild-type plants indicate that the defect is female specific. The genetic and molecular analysis of *AGP18*-silenced plants containing a single T-DNA RNAi insertion suggests that posttranscriptional silencing of *AGP18* is acting both at the sporophytic and gametophytic levels. A cytological analysis of all defective *AGP18*-RNAi lines, combined with the analysis of molecular markers acting at key stages of female gametogenesis, showed that the functional megaspore fails to enlarge and mitotically divide, indicating that *AGP18* is essential to initiate female gametogenesis in Arabidopsis. Our results assign a specific function in plant development to a gene encoding a classical AGP.

### INTRODUCTION

The life cycle of flowering plants consists of a diploid sporophytic phase and two morphologically different haploid gametophytic phases taking place in specialized reproductive organs. Distinct types of meiotically derived cells give rise to the male and female gametophytic phases. In the anther, many microsporocytes develop into pollen grains, which harbor the sperm cells and represent the male gametophyte. In the ovule, usually a single sporophytic cell (the megaspore mother cell [MMC]) undergoes meiosis and gives rise to four haploid products (the megaspores) during a process referred to as megasporogenesis. While three of the megaspores undergo programmed cell death, a single functional megaspore enlarges and gives rise to the female gametophyte (or megagametophyte). In *Arabidopsis thaliana*, female gametogenesis initiates when the single functional megaspore divides mitotically to form an eight-nucleate syncytium. Subsequent cellularization partitions the eight nuclei into seven cells: an egg cell and two synergids at the distal (or micropylar) pole of the female gametophyte, three antipodals at the proximal (or chalazal) pole, and a binucleated central cell whose nuclei

fuse before fertilization. This type of development and organization of the female gametophyte defines the Polygonum type that prevails in >70% of the species examined (Maheshwari, 1950; Willemse and van Went, 1984; Reiser and Fischer, 1993; Drews and Yadegari, 2002). Whereas the fusion of a sperm with the egg cell forms a zygote that subsequently develops into an embryo, fertilization of the binucleated central cell eventually gives rise to the endosperm, a triploid tissue essential for seed viability.

Little is known about the genetic basis and molecular mechanisms that regulate the initiation of female gametogenesis in the ovule. A large collection of both sporophytic and gametophytic mutants defective in female gametophyte development has been identified in Arabidopsis (Schneitz et al., 1997; Christensen et al., 1998; Howden et al., 1998; Grini et al., 1999; Drews and Yadegari, 2002). Whereas sporophytic mutations act at the diploid level and are inherited in a Mendelian 3:1 ratio, gametophytic mutants are poorly transmitted through either one or both types of gametes and exhibit distorted segregation patterns. Many mutants that disrupt meiosis have been isolated (Klimyuk and Jones, 1997; Siddiqi et al., 2000; Yang and Sundaresan, 2000; Reddy et al., 2003), but little is known about other developmental aspects of megasporogenesis. To date, *SPOROCTELESS (SPO)/NOZZLE* is the only gene shown to be required for the initiation of microsporogenesis and megasporogenesis (Schiefthaler et al., 1999; Yang et al., 1999). *SPO* encodes a nuclear protein related to MADS box transcription factors that is expressed during early anther and ovule development. In plants homozygous for *hadad (hdd)* and *proliferata (prf)*, female gametophyte development is arrested at either the two-nucleate or the four-nucleate stage, respectively. Whereas the

<sup>1</sup>To whom correspondence should be addressed. E-mail vielle@ira.cinvestav.mx; fax 52-462-624-58-49.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jean-Philippe Vielle-Calzada (vielle@ira.cinvestav.mx).

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gene responsible for the mutation in *hdd* has yet to be identified (Moore et al., 1997), *PRL* encodes a Mcm7-like licensing factor essential for DNA replication (Springer et al., 1995). Two insertional alleles in *CYTOKININ-INDEPENDENT 1*, a gene encoding a putative Arabidopsis His kinase, were shown to cause nuclear degeneration in the female gametophyte as early as the four-nucleate stage (Christensen et al., 1998; Pischke et al., 2002). Recently, the *NOMEGA* gene was shown to be required for cell cycle progression beyond the two-nucleate stage; *NOMEGA* encodes a putative APC6/CDC16 component of the anaphase promoting complex in Arabidopsis (Kwee and Sundaresan, 2003). Several additional gametophytic mutants that fail to progress beyond the one-nucleate haploid stage have been identified (Christensen et al., 1998), but the corresponding genes have yet to be isolated and characterized.

Numerous studies showing that modifications of plant growth conditions can alter the sporophyte to gametophyte transition (Bell, 1989) or even the whole plant reproductive outcome (Knox, 1967) indicate that the presence of molecular signals that determine the fate of competent cells is fundamental for switching from a sporophytic into a gametophytic developmental pathway. Several regulatory proteins have been shown to have important functions in cell signaling and recognition during plant development. Arabinogalactan proteins (AGPs) are an abundant and heterogeneous class of highly glycosylated Hyp-rich glycoproteins widely distributed in the plant kingdom (Fincher et al., 1974; Clarke et al., 1979; Kreuger and van Holst, 1996; Sommer-Knudsen et al., 1998; Gaspar et al., 2001; Showalter, 2001). The recent characterization of genes encoding different AGP backbones in several species gave rise to the current distinction between classical and nonclassical AGPs (Chen et al., 1994; Du et al., 1994; Mau et al., 1995; Knox, 1999). Classical AGPs contain a domain responsible for attaching the protein backbone to a glycosylphosphatidylinositol (GPI) membrane anchor (Schultz et al., 1998; Youl et al., 1998; Sherrier et al., 1999; Schindelman et al., 2001; Borner et al., 2002; Sun et al., 2004). By contrast, nonclassical AGPs lack the GPI anchor signal and are soluble components of the extracellular matrix often containing Asn- or Hyp-rich domains (Majewska-Sawka and Nothnagel, 2000; Schultz et al., 2000; Gaspar et al., 2001). Molecular and biochemical evidence indicates that AGPs have specific functions during root formation (Willats and Knox, 1996; Casero et al., 1998; van Hengel and Roberts, 2002), the promotion of somatic embryogenesis (Serpe and Nothnagel, 1994; Kreuger and van Holst, 1993, 1996; van Hengel et al., 2001; van Hengel and Roberts, 2002), or the attraction of pollen tubes in the style (Du et al., 1994; Cheung et al., 1995; Wu et al., 1995; Jauh and Lord, 1996; Roy et al., 1998). The use of monoclonal antibodies directed against carbohydrate epitopes provided evidence suggesting that AGPs play an important role during the alternation between sporophytic and gametophytic transitions in the ovule (Pennell and Roberts, 1990; Pennell et al., 1991; McCabe et al., 1997). Although these studies elegantly showed that the establishment of a female reproductive lineage is associated with changes in the distribution of AGP epitopes (Pennell et al., 1992), they did not identify a specific AGP protein or the corresponding gene acting during ovule development or early embryo formation. A few mutations altering the activity of

specific genes encoding AGPs in Arabidopsis have been described. Homozygous plants for *resistant to agrobacterium transformation 1* are resistant to root-dependent transformation via *Agrobacterium tumefaciens* (Nam et al., 1999); however, mutant plants are phenotypically indistinguishable from the wild type, and no developmental defects associated with the mutation have been described. The mutation is caused by a T-DNA insertion within the promoter region of the Arabidopsis *AGP17* gene (Gaspar et al., 2001). A second insertional mutant in a gene encoding a nonclassical AGP (*AGP30*) has recently been shown to be involved in root regeneration and seed germination (van Hengel and Roberts, 2003). Recently, hybrid-type proteoglycans having properties of both AGPs and lipid-transfer proteins have been shown to be essential for the differentiation of tracheary elements in *Zinnia elegans* and Arabidopsis (Motosue et al., 2004).

In this study, we report the enhancer detection-based identification of *AGP18*, a classical AGP gene that specifically acts during female gametophyte development in Arabidopsis. To determine the function of *AGP18*, we introduced double-stranded RNA in wild-type plants and specifically degraded the endogenous *AGP18* transcript by RNA interference (RNAi). More than 75% of the primary transformants resulted in lines showing reduced seed set but no additional developmental abnormalities. Reciprocal crosses to wild-type plants suggested that the defect is female specific. The genetic and molecular analysis of a line containing a single T-DNA RNAi insertion suggests that posttranscriptional silencing of *AGP18* is acting both at the sporophytic and gametophytic levels. The cytological analysis of all defective *AGP18*-RNAi lines indicates that *AGP18* is essential to initiate female gametogenesis in Arabidopsis. Our results assign a specific function in plant development to a gene encoding a classical AGP.

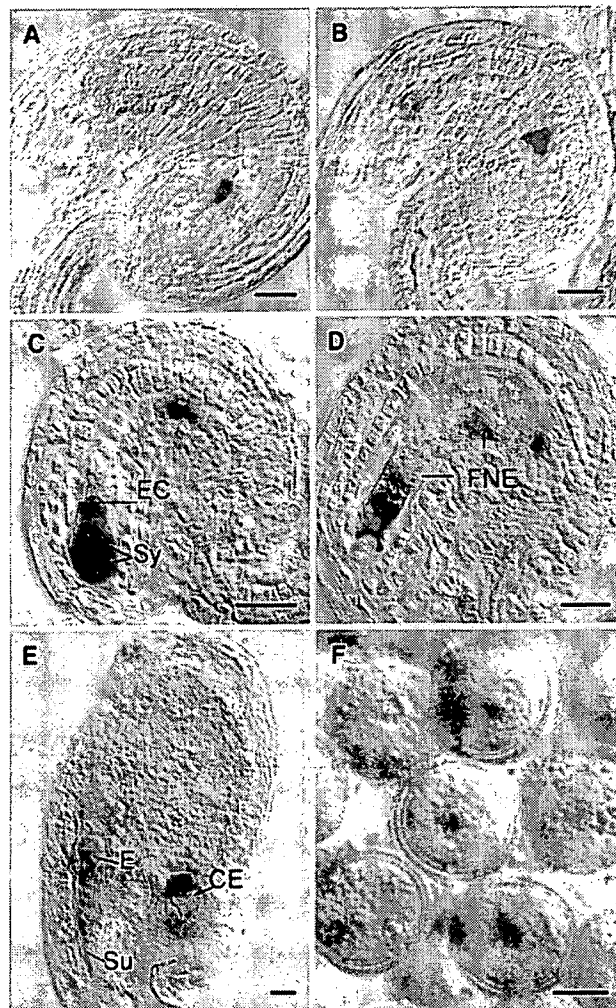
## RESULTS

### Enhancer Detection Tagging of *AGP18*

Using the system established by Sundaresan et al. (1995), we have generated a *Ds* enhancer detector and a gene trap population to identify patterns of expression associated with genes acting during female gametophyte development in Arabidopsis. The enhancer detection vector relies on a maize (*Zea mays*) *Ds* transposon carrying a  $\beta$ -glucuronidase reporter gene (*uidA* or *GUS*) under the control of a minimal promoter. Such a reporter construct is not trapping genes but rather integrating into genomic sequences to serve as a detector of any given regulatory sequence that is acting as an enhancer of promoter activity at the specific location of the insertion (Bellen, 1999; Springer, 2000). The *Ds* enhancer detector element (DsE) also contains the neomycin phosphotransferase II (*NPTII*) gene (conferring resistance to kanamycin); *NPTII* acts as a selectable marker and facilitates the genetic analysis of segregating enhancer detector or gene trap lines.

Whole-mount staining and clearing procedures allow screening for reporter gene expression (*GUS*) at different developmental stages encompassing megasporogenesis and female gametogenesis, from the time when the ovule primordium has just started its elongation (before MMC differentiation) to stages

where the female gametophyte is fully differentiated (J.-P. Vielle-Calzada and U. Grossniklaus, unpublished results; Vielle-Calzada et al., 2000). Figure 1 shows the pattern of GUS expression identified in MET333. MET333 shows initial GUS expression in the chalazal region of the four-nucleate female gametophyte (Figure 1A). At the eight-nucleate stage, expression is restricted to the young antipodal cells and the cellularizing



**Figure 1.** Pattern of GUS Expression in the Enhancer Detector Line MET333.

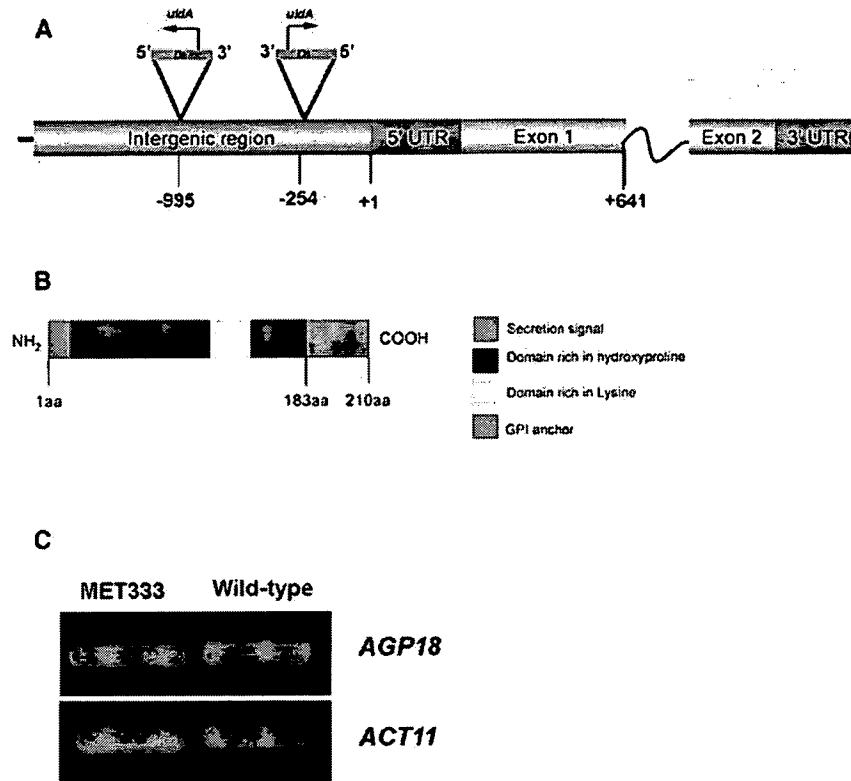
- (A) Female gametophyte at four-nucleate stage.  
 (B) Cellularized female gametophyte.  
 (C) Mature female gametophyte before fertilization.  
 (D) Female gametophyte after fertilization.  
 (E) Embryo at four-cell stage.  
 (F) Mature pollen with GUS expression associated with the vegetative nucleus.  
 Sy, synergids; EC, egg cell; E, embryo; FNE, free nuclear endosperm; Su, suspensor; CE, chalazal endosperm. Bars in (A) to (E) = 20  $\mu$ m; bar in (F) = 10  $\mu$ m.

egg apparatus (Figure 1B). At maturity, the female gametophyte shows GUS expression in the synergids, the egg cell, and the antipodals but not in the central cell (Figure 1C). This pattern of expression persists after fertilization (Figure 1D); however, GUS is also expressed in the free nuclear endosperm after fertilization of the central cell (Figure 1D). During early seed development, GUS is expressed in the embryo proper, the suspensor, and the chalazal endosperm (Figure 1E). Interestingly, MET333 also shows GUS expression in cytoplasmic domains closely associated with the vegetative nucleus of mature pollen grains and in pollen tubes (Figure 1F) but not in microsporocytes at earlier stages of development. A detailed analysis of MET333 plants homozygous for kanamycin resistance did not reveal a mutant phenotype at any stage of plant reproductive development.

DNA gel blot analysis using a DsE-specific probe that includes a portion of *NPTII* showed that two copies of the DsE element were present in MET333 heterozygotes but were absent from wild-type siblings in which GUS expression was not detected (data not shown). Genomic sequences flanking both DsE elements were rescued using thermal asymmetric interlaced PCR (Liu et al., 1995). Sequence analysis of the PCR products showed that the DsE elements were inserted 254 and 995 bp upstream of the transcription initiation site of *AGP18* (At4g37450). Figure 2A illustrates the molecular structure of *AGP18* and the localization of both DsE insertion sites. *AGP18* encodes a classical AGP containing a C-terminal domain responsible for anchoring the protein to GPI. In animals, GPI anchors have been shown to provide an alternative to transmembrane proteins for anchoring proteins to components of the cell surface (Takos et al., 1997, 2000; Schultz et al., 1998; Svetek et al., 1999; Borner et al., 2002; Sharma et al., 2004). Additionally, *AGP18* contains an N-terminal secretory signal predicted to direct the secretion of the protein via the endoplasmic reticulum, two Pro-rich domains that are possible targets of glycosylation (Tan et al., 2003), and a Lys-rich domain predicted to interact with negatively charged molecules (Figure 2B; Gilson et al., 2001; Schultz et al., 2002). The Lys-rich domain is present in only 3 of 15 classical AGPs found to be encoded in the genome of *Arabidopsis* (Schultz et al., 2002). RT-PCR analysis revealed that the levels of *AGP18* transcription in MET333 are similar to the wild type, confirming that *AGP18* expression is not diminished by the presence of the DsE insertions (Figure 2C).

#### ***AGP18* Is Expressed in Adjacent Sporophytic and Gametophytic Cells**

To determine if the pattern of GUS expression identified in MET333 reflected the pattern of expression of *AGP18*, we determined the localization of *AGP18* mRNA by in situ hybridization. To avoid the detection of mRNA corresponding to other AGP transcripts structurally resembling *AGP18*, sense and antisense digoxigenin-labeled probes were generated using a specific portion of the first exon that shows no homology with other AGP genes. The results are summarized in Figure 3. Detailed analysis of all aerial parts of *Arabidopsis* demonstrated that *AGP18* mRNA could be localized only in developing anthers and ovules and transiently in clusters of companion cells closely associated with vascular elements of the stem. *AGP18* is initially



**Figure 2.** Genomic Structure and Protein Organization of *AGP18*.

The enhancer detector line MET333 has two DsE elements inserted in the 5' regulatory region of *AGP18*.

**(A)** Genomic structure of *AGP18*. The arrows show the direction of transcription of *uidA* (GUS).

**(B)** Predicted protein structure of *AGP18*. aa, amino acids.

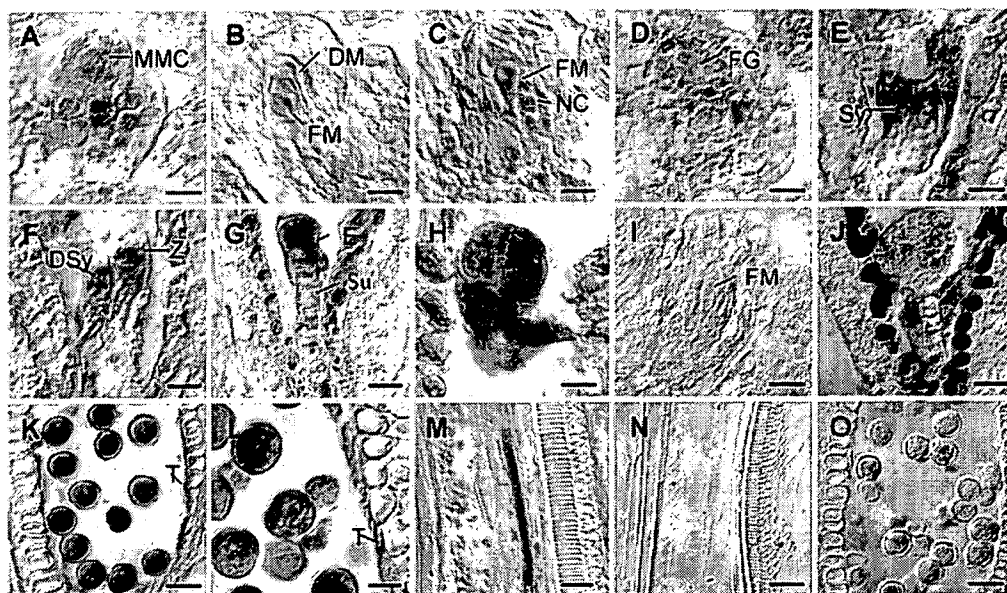
**(C)** RT-PCR analysis shows that the levels of transcription of *AGP18* are identical in MET333 and wild-type plants.

expressed in the MMC and the neighboring nucellar cells of the young ovule primordium (Figure 3A). *AGP18* expression persists in all four products of female meiosis (Figure 3B). At the end of megasporogenesis, when the three nonfunctional megaspores have already degenerated, *AGP18* mRNA is abundant in the functional megaspore but also in the adjacent nucellar cells (Figure 3C). During female gametogenesis, *AGP18* is expressed in the developing female gametophyte (Figure 3D). At maturity, abundant *AGP18* mRNA can be detected in the synergids (Figure 3E), the egg cell, and the antipodals but not in the central cell. After fertilization, *AGP18* mRNA is present in the developing embryo as well as in the free nuclear endosperm (Figures 3F to 3H). Abundant levels of *AGP18* mRNA persist in the embryo until the late globular stage and subsequently start to decrease. No *AGP18* mRNA can be detected in seeds containing torpedo or cotyledonary embryos. In the anther, *AGP18* is expressed in the tapetum and the mature pollen grain (Figures 3K and 3L). These results indicate that GUS expression in MET333 overlaps with the localization of *AGP18* mRNA, confirming that DsE elements partially detect the expression of *AGP18*; however, the absence of GUS expression in MET333 sporophytic cells (the developing nucellus and the mature tapetum) suggests that additional

regulatory elements driving the expression of *AGP18* are not detected by either DsE element.

#### Generation of *AGP18*-RNAi Plants and Analysis of RNA Levels

To determine the role of *AGP18* in Arabidopsis, a 740-bp fragment of the *AGP18* cDNA was cloned into a pFGC5941 RNAi vector (Kerschen et al., 2004) in both sense and antisense orientations and used to transform wild-type Columbia plants. Figure 4A illustrates the RNAi construct that was used to conduct these experiments. pFGC5941 contains a 35S promoter of *Cauliflower mosaic virus* (CaMV35S) that drives the transcription of a partial *AGP18* sequence cloned in both sense and antisense orientations and separated by an intron of the chalcone synthase gene. After formation of hairpin RNA structures, the resulting double-stranded RNA transcripts can cause posttranscriptional silencing of endogenous gene activity (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000; Smith et al., 2000). Although a detailed pattern of CaMV35S promoter activity during male and female gametogenesis has yet to be determined in Arabidopsis, we reasoned that *AGP18* transcripts localized in sporophytic



**Figure 3.** Localization of *AGP18* mRNA by in situ hybridization.

(A) MMC stage. Bar = 9  $\mu$ m.

(B) Female meiosis stage. Bar = 8  $\mu$ m.

(C) Functional megaspore stage with young nucellus. Bar = 8.5  $\mu$ m.

(D) Two-nucleate stage female gametophyte. Bar = 14  $\mu$ m.

(E) Mature female gametophyte. Bar = 8  $\mu$ m.

(F) Zygote stage. Bar = 9  $\mu$ m.

(G) Embryo four-cell stage. Bar = 15  $\mu$ m.

(H) Embryo at early globular stage. Bar = 11  $\mu$ m.

(I) Functional megaspore and young nucellus, sense probe. Bar = 15  $\mu$ m.

(J) Embryo at early globular stage, sense probe. Bar = 20  $\mu$ m.

(K) Mature pollen. Bar = 24  $\mu$ m.

(L) Anther showing the tapetum. Bar = 20  $\mu$ m.

(M) Longitudinal section of a stem. Bar = 20  $\mu$ m.

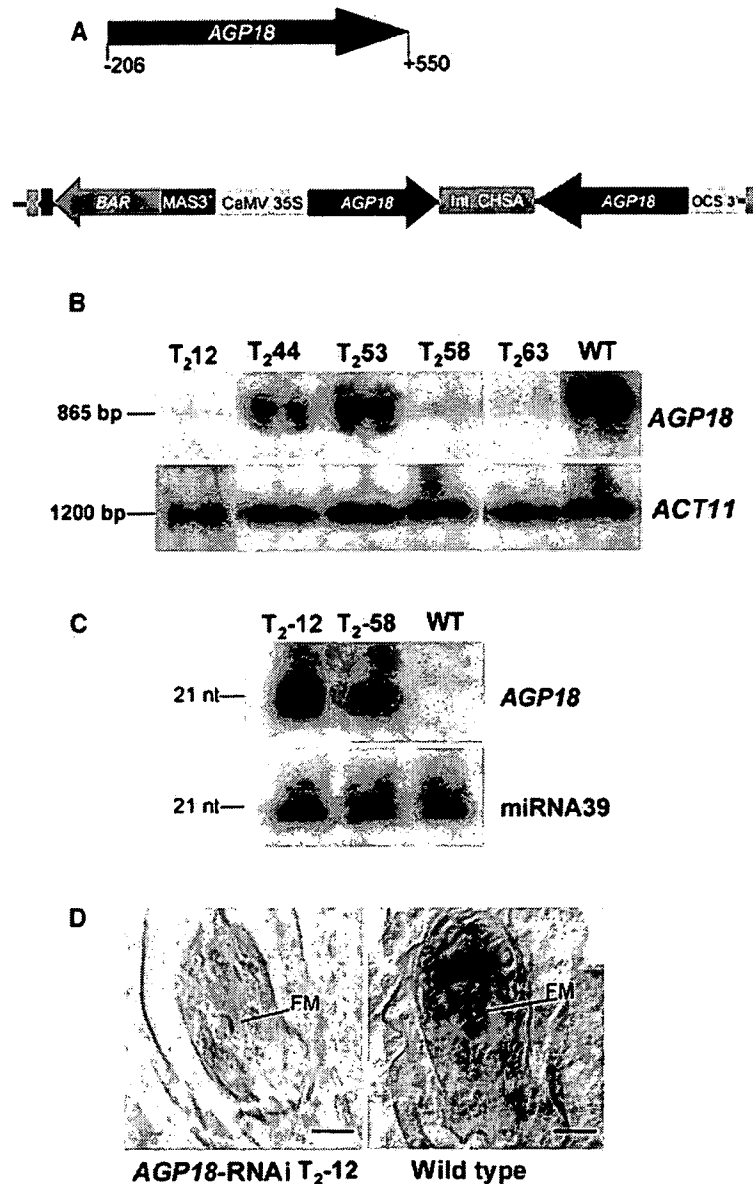
(N) Longitudinal section of a stem, sense probe. Bar = 20  $\mu$ m.

(O) Mature pollen, sense probe. Bar = 20  $\mu$ m.

(A) to (H) and (K) to (M) hybridizations with antisense probe; (I), (J), (N), and (O) hybridizations with sense probe. NC, nucellar cells; FM, functional megaspore; DM, degenerating megaspores; Sy, synergids; DSy, degenerating synergid; FG, female gametophyte; Z, zygote; E, embryo; Su, suspensor; T, tapetum.

cells can be the target of RNAi-dependent silencing driven by CaMV35S. After floral-dipping transformation, 75 primary transformants were generated, none of which showed visible defects during vegetative growth, root development, or floral organogenesis; however, 58 out of 75 adult T1 transformants showed semisterility defects. All 58 transformants maintained a reduced fertility phenotype in the T2 generation. To determine a possible relationship between a decrease in *AGP18* transcript levels and the defective phenotype, RNA was extracted from developing gynoecia of *BASTA*-resistant *AGP18*-RNAi T2 lines and used for RNA gel blot analysis. The results are shown in Figure 4B, with actin as a constitutive control to show that equal amounts of RNA were used. Compared with wild-type plants, all 10 T2 lines tested showed a substantial decrease in the transcripts levels of *AGP18*. Among those lines, T2-12, T2-44, and T2-58 showed significantly >50% reduction in seed set, whereas

T2-63 and T2-53 had a 27.4 and 49.8% reduction, respectively. No correlation was found between the level of *AGP18* expression determined by RNA gel blot analysis and the degree of sterility; this absence of correlation has been documented in previous studies showing that abnormal phenotypes induced by RNAi are not always associated with a detectable decrease in transcript levels (Kerschen et al., 2004; C. Napoly and R. Jorgensen, personal communication). The induction of posttranscriptional gene silencing has been shown to result in the production of 21- to 23-bp RNA fragments with a sequence identical to a portion of the silenced gene (Elbashir et al., 2000). To determine if the production of 21- to 23-bp RNA fragments could be associated with the degradation of the *AGP18* transcript, polyacrylamide gels were used to detect the presence of small RNA fragments corresponding to *AGP18*. As shown in Figure 4C, small RNAs corresponding to *AGP18* were detected in lines in which



**Figure 4.** Accumulation of *AGP18* Transcript, Presence of 21-bp Small RNAs, and Absence of *AGP18* Expression in the Gynoecium of *AGP18*-RNAi T2 Lines.

**(A)** Schematic diagram of the vector used to posttranscriptionally silence *AGP18*. The arrow indicates the sequence cloned in the RNAi silencing vector. Numbers indicate nucleotide positions with respect to initiation of the *AGP18* mRNA.

**(B)** Expression analysis of four *AGP18*-RNAi T2 lines and a wild-type control. RNA was isolated from mature gynoecia in both silenced and wild-type plants. A portion of the *AGP18* cDNA was used as a probe. RNA gel blots were subsequently rehybridized with a specific actin probe (*ACT11*) as a loading control.

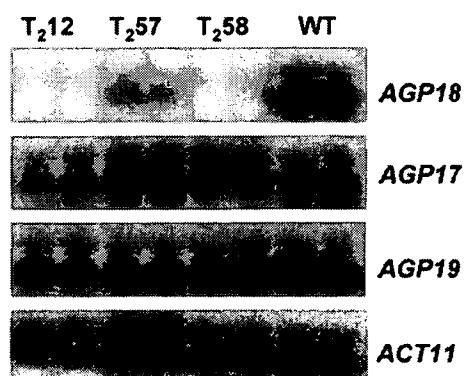
**(C)** A polyacrylamide gel of 100  $\mu$ g of low molecular weight RNA extracted from gynoecia of *AGP18*-RNAi T2 lines and wild-type plants was blotted and hybridized with a portion of the *AGP18* cDNA. The blot was rehybridized with a probe specific to the constitutively expressed microRNA 39 (*miR39*) as a control. nt, nucleotides.

**(D)** Localization of *AGP18* mRNA in developing ovules at the functional megaspore stage. In situ hybridization with specific *AGP18* digoxigenin-labeled antisense probes was performed on gynoecia of both silenced (*AGP18*-RNAi T2-12; bar = 8.5  $\mu$ m) and wild-type plants (bar = 10  $\mu$ m). FM, functional megaspore.

sufficient quantities of total RNA were available, including lines having strong fertility defects as T2-12 and T2-58 but not in wild-type plants. Finally, to determine if a decrease in transcript levels was associated with a decrease in *AGP18* expression within the ovule or the anther, we performed in situ hybridization in selected lines showing the lowest levels of *AGP18* transcript. As shown in Figure 4D, no *AGP18* mRNA could be detected in the young ovule at the functional megaspore stage of *AGP18*-RNAi transformants, indicating that the normal expression of *AGP18* during female gametogenesis is severely impaired in *AGP18*-RNAi lines.

#### Posttranscriptional Gene Silencing Is Specific to *AGP18*

Plant transformation with RNAi vectors targeting a conserved gene family has been shown to often result in simultaneous posttranscriptional gene silencing of several family members. In *Arabidopsis*, 16 genes are predicted to encode the protein backbones of classical AGPs (Schultz et al., 2000). *AGP17* and *AGP19* encode classical AGPs with 56 and 42% amino acid similarity to *AGP18*, respectively. All three proteins are the only *Arabidopsis* AGP members containing a Lys-rich domain. At the DNA level, *AGP17* and *AGP19* share 55 and 52% homology with *AGP18* in the 740-bp cDNA fragment that was used to generate the RNAi construct. To determine if the transcript levels of any of these two genes were also decreased in *AGP18*-RNAi lines, total RNA extracted from developing gynoecia of three T2 *AGP18*-RNAi lines showing decreased levels of *AGP18* mRNA accumulation were used to perform RT-PCR analysis. As shown in Figure 5, both *AGP17* and *AGP19* are expressed in the gynoecia of wild-type plants; however, none of the T2 *AGP18*-RNAi lines analyzed showed a significant decrease in either *AGP17* or *AGP19* expression. In lines T2-12 and T2-58, no amplification signal could be detected after blotting RT-PCR gels and hybridizing with the corresponding *AGP18* probe, confirming that in these lines *AGP18* expression is almost completely silenced. These



**Figure 5.** Posttranscriptional Gene Silencing Is Specific to *AGP18*.

RNA extracted from developing gynoecia of selected *AGP18*-RNAi T2 lines (T2-12, T2-57, and T2-58) was used for cDNA synthesis. PCR amplification was performed with primers specific to *AGP17*, *AGP18*, or *AGP19* (Schultz et al., 2002) using as a template samples corresponding to the same cDNA synthesis. Agarose gels were blotted on nitrocellulose membranes and probed with a corresponding AGP probe. Wild-type cDNA and amplification of *ACT11* were used as positive controls.

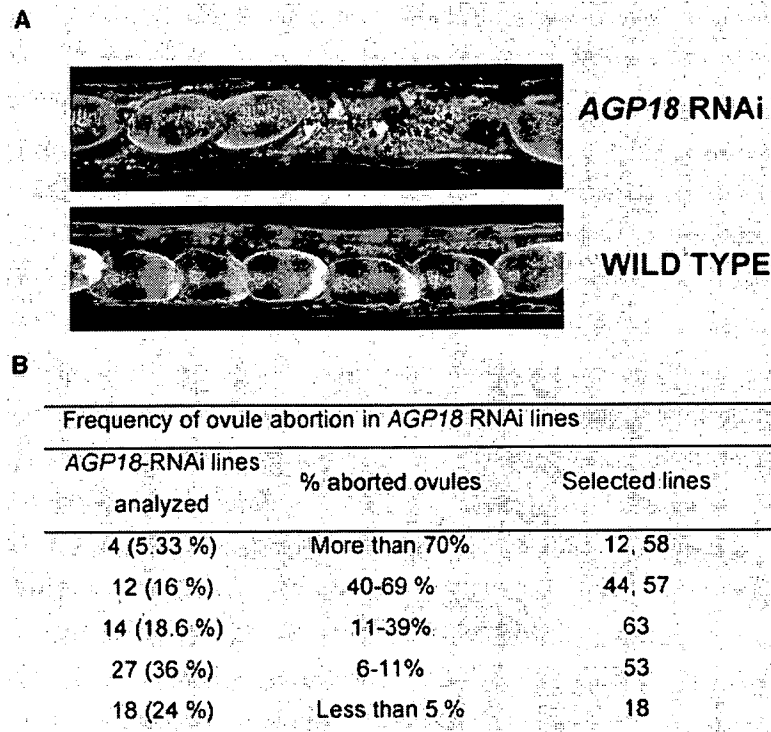
results demonstrate that in *AGP18*-RNAi lines posttranscriptional gene silencing is specific to *AGP18*.

#### Ovule Abortion in *AGP18*-RNAi Lines Is Controlled at the Sporophytic and Gametophytic Levels

As shown in Figure 6A, the siliques of *AGP18*-RNAi T2 lines contain a variable number of aborted ovules that do not show signs of early seed formation. This defect can also be observed in the form of empty spaces within siliques of self-fertilized flowers from *AGP18*-RNAi plants. Gametophytic defects affecting the female gametophyte but not the male are expected to show a decrease in seed set of ~50%. As shown in Figure 6B, T2 lines showed a wide range of frequencies of ovule abortion, with >20% showing a frequency significantly >50% and four lines showing >70% of aborted ovules. To determine the nature of the reproductive defect found in RNAi lines, we conducted reciprocal crosses between lines showing >50% ovule abortion and wild-type plants. When T2 lines were used as female parents, the same percentage of ovule abortion was obtained for all lines tested (data not shown); however, in crosses where T2 lines were used as male parents, full fertility was recovered, suggesting that the sterility defect is female specific.

DNA gel blot analysis was used to determine that the number of RNAi T-DNA insertions present in the genome of T2 lines varied between 1 and 6 (data not shown). Because the T-DNA RNAi construct used is marked with a *BASTA* herbicide resistance marker, its segregation pattern can easily be followed in seedlings. We characterized the segregation of *BASTA* resistance in a group of *AGP18*-RNAi lines showing high levels of ovule abortion but different numbers of T-DNA insertions. A summary of these results are presented in Table 1. Interestingly, a single T-DNA RNAi insertion was present in the genome of T2-12, a line showing 74.9% of aborted ovules and the highest levels of *AGP18* silencing in female reproductive organs. As shown in Table 1, T2-12 segregated *BASTA*-resistant (*BASTA*<sup>r</sup>) and *BASTA*-sensitive (*BASTA*<sup>s</sup>) seedlings in a distorted ratio of 1:1 *BASTA*<sup>r</sup>:*BASTA*<sup>s</sup> ( $\chi^2 = 2.41 < \chi^2_{0.05[1]} = 3.84$ ) as compared with 3:1 expected for normally transmitted insertions. In addition, no homozygous individuals have been identified in the T4 progeny resulting from self-pollination of 20 heterozygous T3-12 plants, suggesting that the transgene is poorly or not transmitted through the gametophytic phase of the *Arabidopsis* life cycle. The frequency of aborted ovules and the distorted segregation ratio in T2-12 suggests that posttranscriptional silencing of *AGP18* is affecting female reproductive development both at the sporophytic and gametophytic levels. In lines containing more than one T-DNA RNAi insertion, the number of *BASTA*<sup>r</sup> seedlings is significantly increased, suggesting that not all insertions are abnormally transmitted through the gametophytic phase and that the degree of semisterility found in *AGP18*-RNAi lines is not directly proportional to the number of introduced T-DNA insertions. These results are in agreement with recent estimations of the efficiency of RNAi in transgenic plants (Kerschen et al., 2004).

To determine if fertility defects were consistently inherited, we quantified the sterility phenotype during the first three consecutive generations of four selected lines. As shown in



**Figure 6.** Siliques of AGP18-RNAi Lines Show Aborted Ovules.

**(A)** Micrographs of AGP18-RNAi and wild-type siliques. The asterisks indicate the aborted ovules observed in AGP18-RNAi lines.

**(B)** An average of 250 ovules was scored for each AGP18-RNAi line. A  $\chi^2$  statistical analysis showed that lines having >5% ovule abortion were significantly different from the wild type (2.5% ovule abortion).

Table 2, the percentage of aborted ovules is maintained in the T3 generation; however, a small decrease in the frequency of ovule abortion was observed in all lines evaluated. Statistical analysis of variance showed that this decrease is not statistically significant during the generations tested.

#### Female Gametophyte Development Is Defective in AGP18-RNAi Lines

To determine the cellular nature of the defect, whole-mounted cleared anthers and ovules were analyzed during male and female reproductive development. We initially determined the terminal phenotypes of all AGP18-RNAi lines by examining mature organs at developmental stages in which male and female gametogenesis have normally resumed and fully differentiated cellularized gametophytes are already formed. All 58 lines examined had a variable proportion of ovules showing a similar abnormal phenotype but no defects in pollen formation. Our observations are summarized in Figure 7. In the large majority of defective ovules, a single conspicuous cell with a centrally located nucleus was present in the nucellus. In all lines, the conspicuous cell did not show signs of degeneration, and the adjacent nucellar tissue was not reabsorbed; in rare cases, its nucleus was not observed. To determine the developmental stage at which female gametophyte development

first departs from the wild type, we analyzed female gametogenesis at earlier developmental stages in ovules of line T2-12 (showing 74.9% of ovule abortion). All ovules of T2-12 undergo normal and synchronized megasporogenesis. Shortly after the differentiation of the functional megaspore (Figures 7A and 7E), a minority of ovules divide mitotically and give rise to two nuclei located at opposite ends of an enlarged cell showing a central vacuole. This type of ovule does not show differences with wild-type development (Figure 7B). By contrast, within the same gynoeceum, the nucellus of the majority of ovules contains a single cell that closely resembles the differentiated functional megaspore but that shows no signs of subsequent enlargement or vacuolization (Figure 7F). At later stages of development, whereas some ovules undergo two additional divisions and form a normal female gametophyte identical to the wild type (Figure 7C), the majority contains a single cell that does not divide mitotically (Figures 7G and 7H). To determine if the frequency of aborted ovules scored in dissected siliques is similar to the frequency of ovules showing abnormal female gametophyte development, we quantified the number of ovules showing a single conspicuous cell in the nucellus of fully differentiated ovules. The results are shown in Table 3. For all lines examined, there is a close correlation between the two values, suggesting that defects in female gametophyte development are sufficient to explain the abnormal phenotype observed in AGP18-RNAi lines.

**Table 1.** Number of T-DNA Insertions, Segregation Analysis, and Percentage of Ovule Abortion in Selected T2 *AGP18*-RNAi Lines

Line	Number of Insertions	BASTA <sup>r</sup>	BASTA <sup>s</sup>	Ratio	Viable Ovules	Aborted Ovules	Percentage of Ovule Abortion
T2-12	1	127	153	1.0:1.2	62	185	74.90
T2-57	2	143	53	2.6:1.0	100	125	55.55
T2-58	2	186	15	12.4:1.0	67	167	71.36
T2-44	5	182	46	3.9:1.0	125	99	49.01

### The Functional Megaspore Does Not Initiate Female Gametogenesis in *AGP18*-RNAi Ovules

To determine the identity of the cell that persists in the nucellus of defective ovules, we took advantage of additional enhancer detection and gene trap lines that show GUS expression in specific cells of the developing female gametophyte. Some of them represent ideal molecular markers to conduct crosses with *AGP18*-RNAi lines showing a high proportion of defective ovules. Figure 8 illustrates the pattern of GUS expression obtained in ovules of F1 plants resulting from crosses of line *AGP18*-RNAi T2-12 with individuals homozygous either for the ET499 or for the ET2209 enhancer detection element. In ET499, GUS is only expressed in the functional megaspore and not in the three dying megaspores or at earlier stages of megasporogenesis (Figures 8A to 8C; J.-P. Vielle-Calzada and U. Grossniklaus, unpublished results). This observation was confirmed in 100 whole-mounted and cleared ovules of homozygous ET499 plants. Other enhancer detection lines show GUS expression in the dying megaspores but not in the functional megaspore (Figure 8D), indicating that ET499 is an appropriate marker to characterize megaspores that have acquired a functional identity at the end of megasporogenesis. By contrast, ET2209 shows GUS expression at the onset of the second haploid mitotic division of the uncellularized female gametophyte and subsequently in all haploid differentiated cells: the synergids, the egg cell, the central cell, and the antipodals (Figure 8G; Vielle-Calzada et al., 2000). In defective *AGP18*-RNAi/+ ET499/+ F1 ovules, GUS expression is restricted to the conspicuous cell that persists in the nucellus after meiosis (Figures 8E and 8F), suggesting that in *AGP18*-RNAi lines female gametophyte development is arrested after the differentiation of the functional megaspore. By contrast, defective *AGP18*-RNAi/+ ET2209/+ F1 do not show GUS expression (Figure 8H), indicating that the arrested functional megaspore does not acquire the identity of a multinucleated female gametophyte or of any of the haploid gametophytic cells. These results indicate that defective *AGP18*-RNAi ovules fail to undergo haploid mitosis after differentiation of the functional megaspore.

**Table 2.** Inheritance of Ovule Abortion in *AGP18*-RNAi Lines

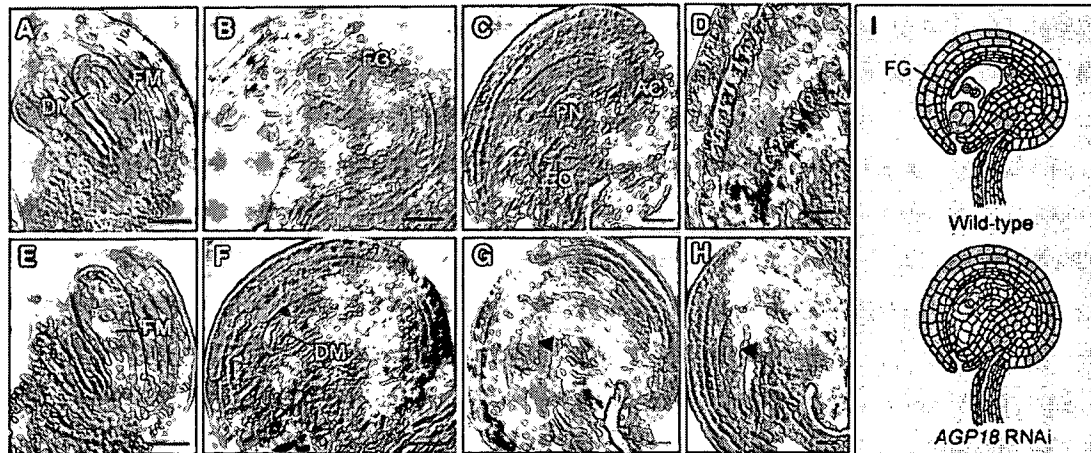
Generation	Line			
	12	44	57	58
T1	74.9% (247)	44.1% (224)	55.50% (225)	71.36% (234)
T2	73.2% (220)	45.2% (271)	54.90% (232)	74.00% (255)
T3	67.5% (234)	39.0% (230)	46.15% (244)	70.80% (222)

### DISCUSSION

Here, we report the successful use of RNAi-induced posttranscriptional silencing to inactivate the *AGP18* gene and show that it plays an essential role during the initiation of female gametogenesis in Arabidopsis. *AGP18* encodes a classical AGP shown to be expressed in cells that spatially and temporally defines the sporophytic to gametophytic transition, but also during early stages of embryogenesis. More than 77% of independent transgenic Arabidopsis lines expressing the *AGP18*-RNAi construct showed moderate to severe fertility defects reminiscent of semi-sterile gametophytic mutants in Arabidopsis. Although in other experiments RNAi-dependent silencing is not always associated with mRNA turnover (Kerschen et al., 2004), all lines tested showed a decrease in *AGP18* transcript accumulation during female reproductive development in the T2 generation. In at least three lines, *AGP18* expression was almost completely suppressed. T2 lines with fertility defects showed ovules impaired in female gametogenesis but normal male gametophytic development and pollen formation. The use of molecular markers expressed at key stages of female gametogenesis determined that in defective ovules meiosis gives rise to a differentiated functional megaspore that is unable to give rise to a two-nucleate female gametophyte.

In plants developing a female gametophyte of the Polygonum type, megasporogenesis ends with the initiation of the haploid phase of the life cycle during the mitotic division of the functional megaspore nucleus (Huang and Russell, 1992). Although little is known about cellular communication during early ovule development, the interaction between sporophytic and gametophytic tissues has been suggested to be essential for female gametogenesis. For example, the isolation of the meiotic precursors and young tetrads by the accumulation of callosic walls has been interpreted as an interfacial reaction leading to the necessary separation of the two generations and the consequent protection of the haploid phase in ferns, mosses, and flowering plants (Dickinson, 1994; Bell, 1995). In Arabidopsis, the deposition of callose in dying meiotic products separates these cells from the functional megaspore (Webb and Gunning, 1990); however, the frequent formation of plasmodesmata connecting the functional megaspore to its adjacent nucellar cells indicates that cell-to-cell communication at the sporophytic-gametophytic transition is important during female gametophyte development (Bajon et al., 1999). In wild-type plants of Arabidopsis, several changes occur during the cytoplasmic maturation of the functional megaspore, including the polarized enlargement of the cell after the micropylar chalazal axis, the formation of a central vacuole, and the concomitant division of the nucleus (Webb and Gunning, 1990;





**Figure 7.** Female Gametophyte Development Is Defective in Ovules of *AGP18*-RNAi Lines.

Wild-type and *AGP18*-RNAi T2 gynoeceia were fixed, cleared, whole mounted, and viewed under Nomarsky optics.

(A) to (D) Development of wild-type ovules.

(A) Functional megaspore in a developing ovule.

(B) Female gametophyte at the two-nucleate stage.

(C) Mature female gametophyte.

(D) Young embryo at the two-cellular stage.

(E) to (H) Development of *AGP18*-RNAi T2-12 defective ovules.

(E) Functional megaspore in the developing T2-12 ovule.

(F) Arrested cell in defective T2-12 mature ovule (arrowhead); normal ovules in the same gynoeceium are at the two-nucleate stage.

(G) Arrested cell in defective T2-12 mature ovule (arrowhead); normal ovules in the same gynoeceium contain a mature female gametophyte.

(H) Arrested cell in T2-12 mature ovule; normal ovules in the same gynoeceium contain seeds undergoing early stages of embryogenesis.

(I) Schematic representation compares a mature wild-type ovule to a mature *AGP18*-RNAi T2-12 defective ovule.

FM, functional megaspore; DM, degenerating megaspore cells; EC, egg cell; AC, antipodal cells; FG, female gametophyte; PN, polar nuclei. Arrowheads indicate the presence of an arrested cell at the one-nucleate stage. Bars = 20  $\mu$ m.

Grossniklaus and Schneitz, 1998; Schneitz, 1999). No signs of cell enlargement or initial vacuolization were detected in arrested functional megaspores of *AGP18*-RNAi plants. In combination with results from expression analysis of molecular markers acting at specific stages of development, these observations indicate that the function of *AGP18* is required after differentiation of the functional megaspore for the initiation of female gametogenesis.

Several interpretations of the functional activity of *AGP18* during ovule development can be proposed based on the analysis of heterozygous *AGP18*-RNAi lines containing a single T-DNA insertion. Although T2-12 shows a 1:1 segregation ratio of *BASTA*<sup>r</sup> to *BASTA*<sup>s</sup> seedlings expected for defective traits causing gametophytic lethality, it also shows >70% of arrested female gametophytes, indicating that the gametophytic activity of RNAi-mediated silencing of *AGP18* is not sufficient to explain the defective phenotype. As shown by in situ hybridization, *AGP18* is expressed in the MMC and the adjacent nucellar cells; therefore, it is possible that, to ensure the initiation of female gametogenesis, the activity of *AGP18* is required in both sporophytic as well as gametophytic cells. Differences on the degree of penetrance of the RNAi effect at the diploid and haploid levels could explain the variable but incomplete sterility shown by all *AGP18*-RNAi lines. Recent studies suggest that the maximal reduction of target transcript levels is obtained in RNAi lines

containing a single T-DNA insertion (Kerschen et al., 2004). Although each target sequence is characterized by an inherent degree of susceptibility to RNAi-dependent silencing, a systematic study to assess the efficiency of posttranscriptional gene silencing in the gametophytic phase has not been conducted. Therefore, it is currently not possible to assess the effectiveness of *AGP18* silencing in the female gametophyte. A second possibility is that key RNA factors generated by RNAi-mediated *AGP18* silencing are produced at the diploid level and meiotically transmitted to a variable number of functional megaspores not carrying a T-DNA RNAi insertion; alternatively, these factors could be transported from adjacent nucellar cells to the functional megaspore via plasmodesmata. Although the transport of mRNA or proteins has not been reported in female meiotic products, detailed ultrastructural studies of megasporogenesis in *Arabidopsis* have shown that multiple plasmodesmata form between the functional megaspore and its adjacent nucellar cells (Bajon et al., 1999). Under this hypothesis, the function of *AGP18* could be strictly gametophytic; however, the non-fully penetrant effect of RNAi-mediated silencing factors generated at the diploid level would be responsible for the abortion of female gametophytes at a frequency significantly higher than 50%. A third alternative includes the possibility that gametophytic lethality in T2-12 results from differences in the degree of RNAi silencing mediated by the CaMV35S promoter. A potential lack

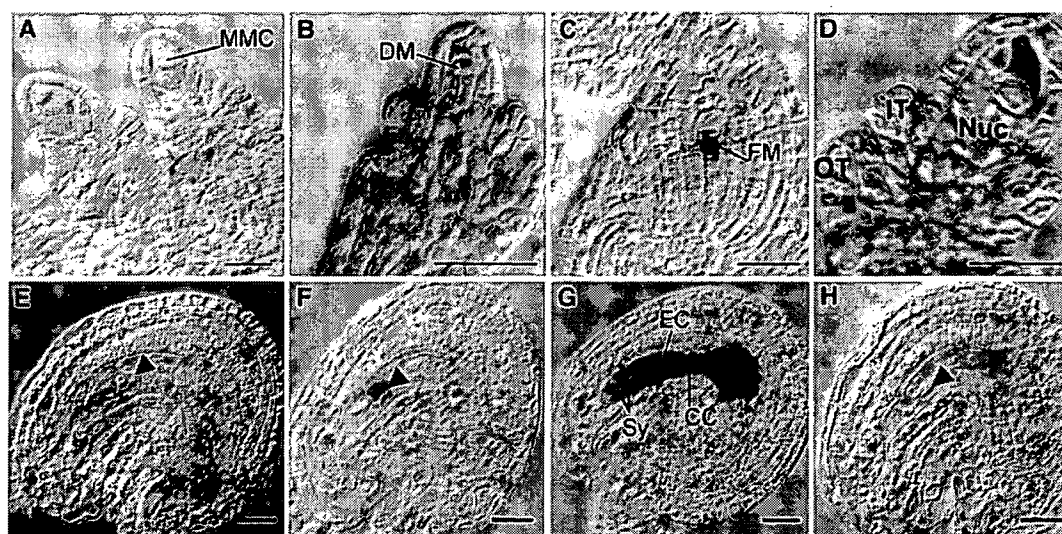
**Table 3.** Frequency of Ovules Showing an Arrested Cell Phenotype

<i>AGP18</i> -RNAi Line	Viable Ovules	Aborted Ovules	Ovules with Arrested Cells
T2-12	138 (27.7%)	185 (74.9%)	359 (72.23%)
T2-57	196 (43.3%)	125 (55.5%)	257 (56.73%)
Wild type	443 (99.4%)	6 (2.4%)	3 (0.60%)

of CaMV35S activity during microsporogenesis could partially explain the absence of pollen abnormalities associated with the expression of *AGP18* during anther development. To date, a detailed pattern of the CaMV35S promoter activity during male and female gametogenesis has yet to be reported in *Arabidopsis*. Although it is generally believed that this promoter is not active during the gametophytic phase (Bechtold et al., 2000), it is not clear at which developmental stage of the sporophytic to gametophytic transition its activity is no longer detected in either male or female gametes. Although this lack of quantitative information on the pattern of CaMV35S complicates the elucidation of the role played by *AGP18* during the alternation of

diploid and haploid phases, experiments showing that RNAi factors transmitted during meiosis can trigger posttranscriptional silencing in the female gametophyte support the hypothesis that the CaMV35S promoter can be used to successfully target gametophytically expressed genes (our unpublished results). The use of specific promoters to drive RNAi-mediated silencing exclusively in the gametophyte or in the nucellus should lead to the elucidation of the specific role of gametophytic and sporophytic *AGP18* activity during the initiation of female gametogenesis.

Classical AGPs play a role in mechanisms as distinct as cell division, cell expansion, or cell determination (Nothnagel, 1997; Schultz et al., 1998; van Hengel and Roberts, 2003). Monoclonal antibodies (MAbs) raised against AGP epitopes have been extensively used to investigate the cellular localization of AGPs (VandenBosch et al., 1989; Knox, 1992); however, these probes do not easily allow the elucidation of single AGP distribution patterns because they can recognize many different AGPs containing a conserved sugar epitope but different protein backbones. Elegant immunolocalization studies have shown that the establishment of a reproductive lineage in certain

**Figure 8.** The Functional Megaspore Does Not Initiate Female Gametogenesis in *AGP18*-RNAi Ovules.

Ovules of F1 plants resulting from crosses of line *AGP18*-RNAi T2-12 with individuals homozygous either for the ET499 or for ET2209 were either fixed and whole-mount cleared or processed for histochemical localization of GUS activity. Shown are patterns of GUS expression in ET499 ([A] to [C]), ET4127 (D), F1 plants resulting from the cross of *AGP18*-RNAi T2-12 and homozygous ET499 plants ([E] and [F]), ET2209 (G), and F1 plants resulting from the cross of *AGP18*-RNAi T2-12 and homozygous ET2209 plants (H).

(A) Ovules of ET499 at MMC not showing GUS expression.

(B) Ovules of ET499 showing absence of expression in all three dying megaspores.

(C) Functional megaspore showing GUS expression in ET499.

(D) Ovule of ET4127 showing GUS expression in a dying megaspore.

(E) Whole-mounted cleared ovule showing the phenotype observed in defective *ET499;AGP18-RNAi* T2-12 F1 ovules.

(F) GUS expression in the arrested functional megaspore of defective *ET499;AGP18-RNAi* T2-12 F1 ovules. The arrowhead shows the position of the arrested functional megaspore.

(G) Pattern of GUS expression in the mature female gametophyte of ET2209.

(H) Absence of GUS expression in defective *ET2209;AGP18-RNAi* T2-12 F1 ovules.

FM, functional megaspore; IT, inner integument; OT, outer integument; Nuc, nucellus; DM, dying megaspore; EC, egg cell; Sy, synergid cells; CC, central cell. The arrowheads indicate the arrested cells. Bars = 20  $\mu$ m.

species was associated with changes in the distribution of AGP epitopes (Knox et al., 1989; Pennell et al., 1992). In *Pisum sativum*, the determination of reproductive cells in male and female gametes is associated with the loss of a cell surface arabinose-containing epitope recognized by the Mab MAC207 (Pennell and Roberts, 1990). Interestingly, a second AGP epitope recognized by Mab JIM8 became detectable only during differentiation of anthers and ovules in *Brassica napus* (Pennell et al., 1991). During ovule development, this epitope was initially detected in the wall of the two- to four-nucleate female gametophyte, in nucellar cells adjacent to the developing female gametophyte, and later in the plasma membrane of all cells forming the egg apparatus, but not the central cell. The presence of this epitope was also detected in the tapetum, in developing pollen grains, and in the stem vasculature (Pennell et al., 1991). Our results show that *AGP18* is expressed in reproductive tissues, particularly in cell types that are involved in establishing the sporophytic to gametophytic transitions. During female gametophyte development, *AGP18* is initially expressed in the MMC, in all four meiotically derived megaspores, the functional megaspore, and the adjacent nucellar cells. *AGP18* expression persists in all female gametophyte cells except the central cell. During male gametophytic development, *AGP18* is expressed in pollen grains and the cells of the tapetum. Developing seeds also express *AGP18* during the first stages of embryogenesis; interestingly, abundant *AGP18* mRNA is detected at early stages of endosperm development, indicating that transcription in the central cell occurs only after double fertilization. We only detected additional *AGP18* expression in restricted clusters of companion cells present in the vasculature of the stem. The pattern of *AGP18* mRNA localization is almost identical to the pattern of localization of the epitope recognized by JIM8, strongly suggesting that an AGP encoded by a gene homologous to *AGP18* is detected by JIM8 in *B. napus*. The generation of *AGP18*-RNAi lines opens new possibilities for immunolocalization analysis with JIM8 and new antibodies raised against *AGP18* to further elucidate the function and distribution of AGPs during reproductive development.

Our results indicate that *AGP18* plays a crucial role during interactions between sporophytic and gametophytic cells in the young ovule and that these interactions are essential for the establishment of the female gametophytic phase in Arabidopsis. Although the nature of this communication has yet to be characterized at the genetic and molecular levels, the elucidation of the developmental function of a gene encoding a classical AGP creates new perspectives for the understanding of cell surface signaling and the molecular mechanisms that regulate sexual reproduction in flowering plants.

## METHODS

### Plant Material and Growth Conditions

The transposant line MET333 (*Arabidopsis thaliana* Heynh. var Landsberg *erecta*) was identified in a collection of enhancer detector lines generated in our laboratory, using the system implemented by Sundaresan et al. (1995), looking for expression patterns during female gametophyte de-

velopment. To select plants carrying the *Ds* transposon, 50 mg/L of kanamycin and 0.66 µg/mL of 1-naphthalenacetamide were added to MS solid medium (Sigma, St. Louis, MO). Resistant seedlings were transferred to soil and grown in a greenhouse under long-day conditions.

### Generation of RNAi Lines

A 740-bp fragment containing the first exon and a region of 5' untranslated region of *AGP18* was amplified by RT-PCR using RNA extracted from wild-type developing gynoecia using the following primers containing restriction sites as indicated in boldface: 5'-**AATCTA-GAGGCGCGCCACGGCTACATCTGTCTGT**-3' (*Xba*I and *Asc*I; sense primer) and 5'-**AAGGATCCATTAAATAATGTACCTGATCGTCGG**-3' (*Bam*HI and *Swa*I; antisense primer). The PCR fragment generated with the sense/antisense primer combination was cloned in pCRII TOPO (Invitrogen, Carlsbad, CA) and subsequently digested with *Swa*I and *Asc*I restriction enzymes. The resulting DNA fragment was cloned using appropriate restriction sites in the silencing vector pFGC5941 (kindly donated by Carolyn Napoli and Rich Jorgensen, www.chromdb.org). The PCR fragment cloned in pCRII TOPO was digested with *Xba*I and *Bam*HI (to obtain the antisense fragment) and cloned in appropriate restriction sites of pFGC5941. The resulting pFGC5941 vector contained *AGP18* in both sense and antisense orientations separated by a chalcone synthase intron and under the control of the CaMV35S promoter. Four-week-old Arabidopsis plants (Columbia-0) were transformed by floral dipping as previously described (Clough and Bent, 1998). Seeds from *Agrobacterium tumefaciens*-treated plants were selected and directly grown under greenhouse long-day conditions (16 h light). Resistant seedlings were selected by spraying the herbicide BASTA (50 mg/L; Finale; AgrRvo, Montvale, NJ) three times each week for 2 weeks. Presence of *AGP18*-RNAi insertions was confirmed by PCR amplification on DNA extracted from seedlings. Seeds from mature plants were collected and plated onto MS medium supplemented with glufosinate ammonium (10 µg/mL; Crescent Chemical, Augsburg, Germany).

### RNA Analysis

For RNA gel blots, total RNA was extracted from developing gynoecia from selected *AGP18*-RNAi transformants and wild-type plants using Trizol (Invitrogen) and following the manufacturer's instructions. Fifty micrograms of RNA were separated in a 1.3% agarose gel containing 17% formaldehyde and blotted onto Hybond N<sup>+</sup> membranes. Blots were hybridized with random-primed (Amersham Biosciences, Buckinghamshire, UK) <sup>32</sup>P-labeled 810-bp probe corresponding to the complete *AGP18* cDNA (At4g37450) and an *ACT11* (At3g12110) probe generated by PCR using the following primers: ACT11-S (5'-TTCAACACTCCTGCCATG-3') and ACT11-AS (5'-TGCAAGGTCCAAACGCAG-3'). The temperature of hybridization was 65°C in Church's buffer.

For small RNA analysis, total RNA from developing gynoecia was enriched for low molecular weight RNAs using Hamilton's homogenization solution (Hamilton and Baulcombe, 1999) as described in Mette et al. (2000). Low molecular weight RNA was normalized by spectrophotometry to 100 µg/lane, separated by electrophoresis through 15% polyacrylamide, 7 M urea, 0.5× Tris-borate EDTA gel, and transferred to Zeta-Probe GT membranes (Bio-Rad, Hercules, CA). After transfer, membranes were cross-linked with 200 mJ of UV and baked at 80°C for 1 h. To detect small RNAs in *AGP18*-RNAi lines, an *AGP18* cDNA probe as described above was randomly labeled and hybridized at 62°C in Church's buffer. An oligonucleotide probe corresponding to the sequence of miR39 (5'-GATATTGGCGCGGCTCAAGCA-3'; Llave et al., 2002) was 5'-end labeled with [<sup>32</sup>P]ATP and hybridized as a loading control. For RT-PCR analysis, total RNA was isolated from developing gynoecia of wild-type and *AGP18*-RNAi lines using Trizol following the manufacturer's instructions. First-strand cDNA was synthesized using

2 µg of total RNA and Superscript II reverse transcriptase (Invitrogen). For semiquantitative RT-PCR, 3 µL of the first-strand cDNA reaction served as a template in a PCR reaction that used specific primers for *AGP18* (At4g37450), *AGP17* (At2g23130), *AGP19* (At1g68725), and *ACT11*. After estimating the amount of amplified DNA produced at different rounds of PCR cycles, we determined that 20 cycles ensured that the amplified product was proportional to the initial concentration of template present in the reaction. After electrophoresis on a 1% agarose gel and blotting into hybrid N<sup>+</sup> membranes, hybridization was performed with <sup>32</sup>P cDNA probes specific to each AGP gene tested and labeled with the random primer method (Amersham Biosciences). Hybridization was performed at 62°C as described (Maniatis et al., 1989). *AGP17* primers were as follows: sense (5'-GCTTTTAAGCCCGCTGCTCC-3') and antisense (5'-CTG-AATACAAATGTGAGCTG-3'). *AGP19* primers were as follows: sense (5'-AAGTTGCACCAAGTAATCAGCC-3') and antisense (5'-TCCTTTAAG-CTGATTTAAGGC-3'). *AGP18* primers were as follows: sense (5'-CACGCTTGTTAACTCC-3') and antisense (5'-TTTTTCATCACT-GACAG-3').

#### Whole-Mount Preparations and Histological Analysis

Wild-type and *AGP18*-RNAi siliques were dissected longitudinally with hypodermic needles (1-mL insulin syringes) and fixed with FAA buffer (50% ethanol, 5% acetic acid, and 10% formaldehyde), dehydrated in increasing ethanol concentration, cleared in Herr's solution (phenol: chloral hydrate:85% lactic acid:xylene:oil of clove [1:1:1:0.5:1]), and observed on a Leica microscope (Wetzlar, Germany) under Nomarski optics. GUS staining assays for stages before fertilization were conducted as described by Vielle-Calzada et al. (2000). For developmental stages after fertilization, we used the protocol described by Köhler et al. (2003) with slight modifications. Longitudinally dissected siliques were fixed for 2 h at -20°C in 90% acetone and subsequently immersed in GUS staining buffer (10 mM EDTA, 0.1% Triton, 0.5 mM Fe<sup>2+</sup>/CN, 0.5 mM Fe<sup>3+</sup>/CN, 100 µg mL<sup>-1</sup> chloranphenicol, and 1 mg mL<sup>-1</sup> 5-bromo-4-chloro-3-indolyl-β-D-galactoside in 50 mM sodium phosphate buffer, pH 7.0) for 1 to 3 d at 37°C. The tissue was cleared in Hoyer's solution and observed under Nomarski optics.

#### In Situ Hybridization

Developing flower buds, developing flowers, isolated gynoecia, and siliques of wild-type and *AGP18*-RNAi T2-12 plants were fixed in 4% paraformaldehyde and embedded in Paraplast (Fisher Scientific, Fair Lawn, NJ). Sections of 12-µm thickness were cut using a Leica microtome and mounted on ProbeOnPlus slides (Fischer Biotech, Pittsburgh, PA). A fragment of 180 bp that included a portion of the first exon of *AGP18* was amplified using sense (5'-CGACGATCAGGTACATTAG-3') and the antisense (5'-CATCACTGACAGATATGAA-3') primers and subsequently cloned in the pCRII TOPO vector (Invitrogen). The resulting construct was digested with *NotI* and *BamHI* to synthesize sense and antisense digoxigenin-labeled probes, respectively, and hybridization was conducted as described by Vielle-Calzada et al. (1999).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number NM119909 (At4g37450).

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# Exhibit 13

*The Plant Journal* (2003) 34, 383–392

## TECHNICAL ADVANCE

# A chemical-regulated inducible RNAi system in plants

Hui-Shan Guo<sup>1</sup>, Ji-Feng Fei<sup>1</sup>, Qi Xie<sup>1</sup> and Nam-Hai Chua<sup>2,\*</sup>

<sup>1</sup>Laboratory of Molecular Cell Biology, Temasek Life Sciences Laboratory, National University of Singapore, 1 Research Link, 117604 Singapore, and

<sup>2</sup>Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

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\*For correspondence (fax +1 212 327 8327; e-mail chua@mail.rockefeller.edu).

### Summary

Constitutive expression of an intron-containing self-complementary 'hairpin' RNA (ihpRNA) has recently been shown to efficiently silence target genes in transgenic plants. However, this technique cannot be applied to genes whose silencing may block plant regeneration or result in embryo lethality. To obviate these potential problems, we have used a chemical-inducible Cre/loxP (CLX) recombination system to trigger the expression of an intron-containing inverted-repeat RNA (RNAi) in plants. A detailed characterization of the inducible RNAi system in transgenic *Arabidopsis thaliana* and *Nicotiana benthamiana* plants demonstrated that this system is stringently controlled. Moreover, it can be used to induce silencing of both transgenes and endogenous genes at different developmental stages and at high efficiency and without any detectable secondary affects. In addition to inducing complete silencing, the RNAi can be produced at various times after germination to initiate and obtain different degrees of gene silencing. Upon induction, transgenic plants with genetic chimera were obtained as demonstrated by PCR analysis. Such chimeric plants may provide a useful system to study signaling mechanisms of gene silencing in *Arabidopsis* as well as other cases of long-distance signaling without grafting. The merits of using the inducible CLX system for RNAi expression are discussed.

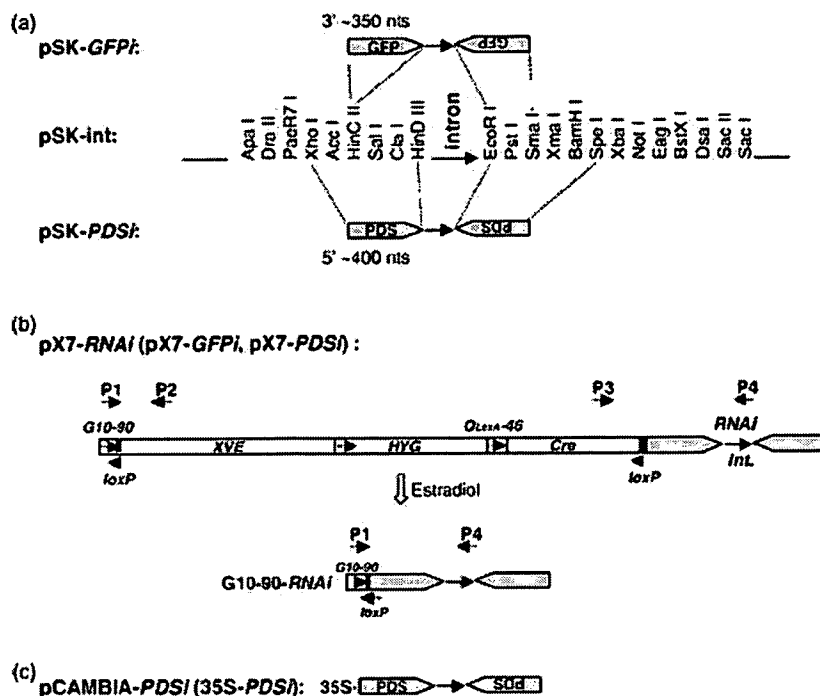
**Keywords:** inducible, constitutive, RNAi, dsRNA, DNA excision, Cre/loxP.

### Introduction

RNA-mediated gene silencing is a conserved mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger sequence-specific degradation of homologous mRNA. dsRNA has been used as a powerful tool for the investigation of RNA silencing in a variety of organisms, such as RNA interference (RNAi) in *Caenorhabditis elegans* (Fire *et al.*, 1998) and mammalian cells (Paddison *et al.*, 2002; Sui *et al.*, 2002) and post-transcriptional gene silencing (PTGS) in plants (Chuang and Meyerowitz, 2000; Waterhouse *et al.*, 1998). In plants and *C. elegans*, RNA silencing involves two steps: (i) a local induced silencing, including an initial processing of the triggering dsRNA into short interfering RNA (siRNA) of 21–25 nt (Elbashir *et al.*, 2001; Hamilton and Baulcombe, 1999) and (ii) a systemic spread of the silencing signal throughout the entire organism (Voinnet *et al.*, 2000; Winston *et al.*, 2002). The presence of a spliceable intron in the transgene encoding the dsRNA appears to enhance the silencing efficiency (Smith

*et al.*, 2000; Wesley *et al.*, 2001). Constitutive expression of intron-containing self-complementary 'hairpin' RNA (ihpRNA) constructs can induce PTGS with almost 100% efficiency when directed against viruses or endogenous genes (Smith *et al.*, 2000).

The completion of the sequencing of the *Arabidopsis* genome has uncovered a large number of genes with unknown functions. Potentially, the dsRNA-mediated gene silencing technique can be used to investigate the functions of these genes. The most effective silencing, brought about by intron-containing dsRNA, would produce phenotypes resembling those of the null alleles of the target genes. If the target gene is required for basic cell function or development, constitutive dsRNA-mediated silencing of the gene may produce detrimental effects or even cause plant lethality resulting in no recovery of transgenic plants for investigation. This problem can be circumvented somewhat by inducing gene silencing, using either *Agrobacterium*



**Figure 1.** Schematic diagrams of the inducible and constitutive RNAi constructs.

(a) A schematic diagram of an intron-containing intermediate vector pSK-int with multiple restriction sites on both arms of the intron. DNA fragments encoding sense and antisense RNA (approximately 350 nt of 3' *GFP* or approximately 400 nt of 5' *PDS*, respectively) were cloned into pSK-int at 5' and 3' arms of the intron in appropriate restriction sites indicated by dotted lines, resulting in pSK-GFPi and pSK-PDSi.

(b) A schematic diagram showing structural features of the inducible pX7-RNAi construct and Cre/loxP-mediated DNA recombination (see Zuo *et al.*, 2001 for details). XVE, a chimeric transactivator containing the regulator domain of an estrogen receptor (Zuo *et al.*, 2000); HYG, a hygromycin-resistance marker; Cre, the bacteriophage P1 Cre recombinase with an intron (Zuo *et al.*, 2001); loxP, specific recognition sites of Cre; OlexA-46, eight copies of the LexA DNA binding site fused to the -46 CaMV 35S promoter; G10-90, a strong, synthetic, constitutive promoter; int, actin 11 intron (ATU27981, nt 1957–2111); RNAi, DNA sequences encoding the intron-containing inverted-repeat RNAs; G10-90-RNAi, the reconstituted transcription unit derived from Cre/loxP-mediated DNA recombination after inducer treatment. Arrows inside transcription units indicate the direction of transcription. P1–4 denote primers used for PCR analysis shown in Figure 5(c).

(c) A fragment of PDS-int-PDS was cloned between the 35S promoter and the terminator in the binary plasmid pCAMBIA 1300 (AF234296) to give 35S-PDSi, a constitutive RNAi construct.

infiltration (Voinnet and Baulcombe, 1997) or virus-derived vectors (Dalmay *et al.*, 2000; Peele *et al.*, 2001; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002). However, these two methods have limited application in functional genomics because of their transient nature, and their silencing effects are not heritable.

In this paper, we describe the development of an inducible gene silencing system using intron-containing, inverted-repeat RNA (referred to as inducible RNAi). The chemical-inducible system we use here is the CLX system, an XVE-based (XVE for LexA-VP16-ER) (Zuo *et al.*, 2000), site-specific DNA recombination mediated by Cre/loxP (Zuo *et al.*, 2001). The Cre expression is placed under the control of the chimeric transcription factor XVE, whose activity is strictly regulated by estrogens. Upon induction by 17 $\beta$ -estradiol, Cre/loxP-mediated recombination leads to activation of the RNAi transcription cassette by bringing it immediately downstream of the constitutive G10-90 promoter (Zuo *et al.*, 2001; Figure 1). Compared to other inducible systems, the most significant feature of the CLX system is that it is stringently controlled, and upon induction, it

produces DNA recombination with high efficiency (for a review, see Hare and Chua, 2002; Ow, 2001).

Here, we show that the inducible RNAi system can be used to silence, with high efficiency, the expression of a *GFP* transgene and an endogenous phytoene desaturase (*PDS*) gene in *Nicotiana benthamiana* as well as in *Arabidopsis thaliana*. Upon induction at seed germination or post-germination stages, the efficiency and effectiveness of *PDS* silencing are comparable to those obtained with a 35S-RNAi construct. A stable and reproducible inducible RNAi phenotype was obtained in subsequent transgenic generations. The merits of the inducible RNAi system for silencing of endogenous genes are discussed.

## Results

### Silencing of a *GFP* transgene

To evaluate the function of the inducible RNAi system, the construct pX7-GFPi (Figure 1b) was introduced into a



transgenic *N. benthamiana* line carrying a 35S-*GFP* transgene (*GFP*-16c) (Ruiz *et al.*, 1998) by *Agrobacterium*-mediated transformation. T<sub>1</sub> seeds from 12 transgenic lines (*GFP*-16c/pX7-*GFPi*, named as 16c-*GFPi*) were germinated on the inductive medium (MS + 2 µM 17β-estradiol) containing hygromycin (hyg). After 2 weeks, eight lines showed uniform red fluorescence under UV light, which was caused by chlorophyll autofluorescence in the absence of *GFP* accumulation. Some red seedlings stopped growing upon continued incubation on the selective medium, presumably because of the complete *Cre/loxP*-mediated excision of the hygromycin-resistance gene upon induction. The remaining four lines initially displayed a mixture of red/green fluorescence after 2 weeks of treatment with the inducer, but propagation of the red color to the entire plant was observed in these lines 2 weeks after they were transferred from the inductive medium to the soil (Figure 2a, left). No difference was observed between 16c-*GFPi* seedlings germinated on the selective medium in the absence of the inducer (MS + hygromycin) and *GFP*-16c control plants, as judged by the degree of green fluorescence (Figure 2b, right).

Northern blots were analyzed with total RNA from treated red fluorescent 16c-*GFPi* seedlings, untreated green fluorescent 16c-*GFPi* seedlings and *GFP*-16c control seedlings. *GFP* mRNA was almost undetectable in red 16c-*GFPi* seedlings (Figure 2b, upper panel, +), as compared with *GFP*-16c control (0) or untreated 16c-*GFPi* seedlings (-). *GFP*-related siRNA, which is a key component of RNA silencing, was detected in red seedlings using a radiolabeled *GFP*-specific probe (Figure 2b, lower panel, +), whereas no *GFP*-related siRNA could be detected in *GFP*-16c control or untreated 16c-*GFPi* green seedlings (0 and -). Both fluorescence and RNA analyses indicated silencing of the *GFP* transgene in 16c-*GFPi* plants upon treatment with 17β-estradiol.

A transgenic line of *A. thaliana* (ecotype C24), which showed constitutive expression of a 35S-*GFP* transgene (Dalmay *et al.*, 2000) was transformed with the pX7-*GFPi* construct to test the inducible RNAi system. Twenty-nine T<sub>1</sub> independent lines (named as At-*GFPi*) were obtained by floral dip transformation. Upon germination on the inductive medium, all 29 At-*GFPi* lines displayed red fluorescence, indicating silencing of the *GFP* transgene. By contrast, all seedlings germinated on the medium without the inducer showed uniform green fluorescence, indicating *GFP* expression (data not shown). Northern blots were analyzed with total RNA (Figure 2c, upper panel) and *GFP*-related siRNA (Figure 2c, lower panel) from treated, red fluorescent At-*GFPi* seedlings (Figure 2c, +) and untreated, green fluorescent At-*GFPi* seedlings (Figure 2c, -). Similar results were obtained as with the *N. benthamiana* 16c-*GFPi* plants, providing molecular evidence for silencing of the *GFP* transgene in At-*GFPi* plants upon 17β-estradiol treatment.

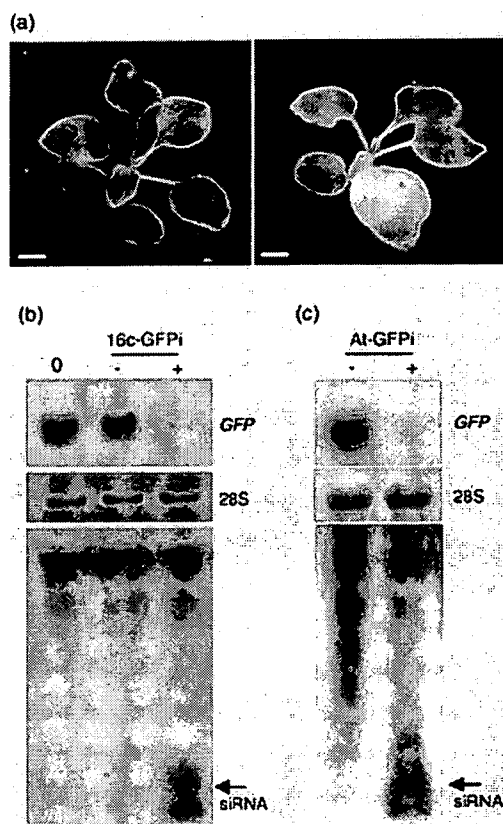
We used 15 At-*GFPi* lines for further investigation of 17β-estradiol-induced silencing at post-germination stage. Two-week-old T<sub>1</sub> seedlings germinated on the selective medium in the absence of the inducer were transferred to fresh MS medium. All seedlings at this stage continued to display green fluorescence. However, when seedlings were transferred to the inductive medium, 10 lines displayed strong inducible *GFP* silencing as indicated by their uniform red fluorescence after 1-week induction (data not shown). The remaining five lines showed varying initiation of *GFP* silencing after 1-week induction. Further incubation with the inducer up to 2 weeks resulted in complete *GFP* silencing as reflected by the uniform red fluorescence in all these plants.

#### *Efficient inducible silencing of an endogenous PDS gene in Arabidopsis thaliana*

We chose the phytoene desaturase (*PDS*) gene of *A. thaliana* and *N. benthamiana* to test the ability of the inducible RNAi system to silence endogenous genes. The *PDS* gene was selected because loss of the phytoene desaturase enzyme blocks carotenoid synthesis culminating in a photobleaching phenotype because of photo-oxidation of chlorophylls (Ruiz *et al.*, 1998). This visible phenotype facilitated visual monitoring of the induction process of *PDS* silencing. The constructs pX7-*PDSi*(At) and pX7-*PDSi*(Nb) (Figure 1b) were transformed into *A. thaliana* (ecotype Columbia) and *N. benthamiana*, respectively. In addition, pCambia-*PDSi*(At) (Figure 1c) containing a 35S-*PDSi*(At) was also transformed into *A. thaliana* (ecotype Columbia).

We tested 35 lines of putative transgenic *A. thaliana* carrying the 35S-*PDSi*(At) transgene by virtue of their ability to grow on the selective medium. Thirty-two lines (35S-*PDSi*(At)) displayed the photobleaching phenotype. Most lines appeared near-white and stopped growing (data not shown) after 4–6 weeks on the culture medium. Only two lines that displayed varying green patches in their bleached leaves survived. Seedlings of these two lines exhibited abnormal development and poor fertility, and produced only a small amount of seeds.

Eighty-one independent *A. thaliana* transgenic lines transformed with pX7-*PDSi*(At) were obtained. In the absence of the inducer, all transgenic T<sub>1</sub> lines (At-*PDSi*) displayed normal development and fertility. T<sub>2</sub> seeds from 12 independent T<sub>1</sub> lines were germinated on the selective medium in the absence or presence of the inducer. All T<sub>2</sub> seedlings grew with normal phenotype on the medium in the absence of the inducer (Figure 3a). However, in the presence of the inducer, seedlings of all the 12 T<sub>2</sub> At-*PDSi* lines showed uniform photobleaching phenotype in the cotyledons at 6–9 days post-induction (Figure 3b,c). Similar to the 35S-*PDSi*(At) lines, most of these seedlings

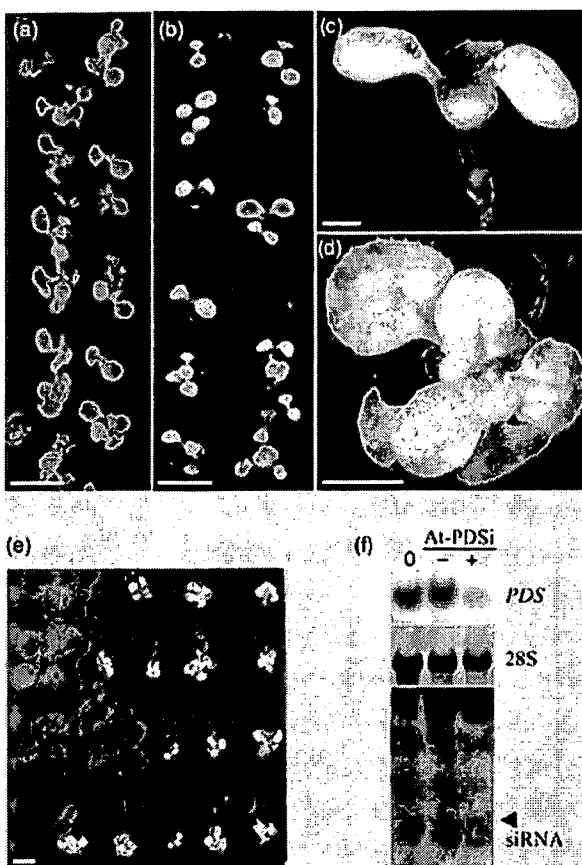


**Figure 2.** 17 $\beta$ -estradiol-inducible GFP silencing in *Nicotiana benthamiana* transgenic line 16c-GFPi and *Arabidopsis thaliana* line At-GFPi.

(a) 16c-GFPi plants were first germinated on an inductive (left) or selective (right) medium for 2 weeks, before being transferred to soil, and photographed under UV light at 2 weeks after transfer. Left, an induced 16c-GFPi plant exhibited uniform red fluorescence in the whole plant. Note that in this plant initiation of GFP silencing was indicated by a mixture of red/green fluorescence. Right, an untreated 16c-GFPi plant showing green fluorescence. Scale bar = 0.5 cm.

(b, c) Northern blot analysis of induced silencing in 16c-GFPi plants (b) and At-GFPi plants (c). Total high (top and middle panels) and low (lower panel) molecular weight RNAs were extracted from 2-week-old red fluorescent seedlings after 17 $\beta$ -estradiol induction (+), untreated green fluorescent seedlings (-), and GFP-16c control plants (0). Equal RNA loading (5  $\mu$ g per leaf) was monitored by methylene blue staining of the 28S RNA (middle panel), and the blot was probed with a GFP-specific sequence (top panels). Bottom panels show the blot for siRNA, which was hybridized with a <sup>32</sup>P-labeled transcript corresponding to the 3'-terminal region (350 nt) of GFP. Each lane contained 50  $\mu$ g RNA. Arrow indicates the position of a 25-base DNA oligonucleotide.

stopped growing within 4–6 weeks (Figure 3d). Northern analysis showed that endogenous *PDS* mRNA levels were significantly reduced in bleached leaves of treated At-PDSi lines (Figure 3f, upper panel, +), but readily detected in untreated lines (Figure 3f, upper panel, -) and WT control seedlings (Figure 3f, upper panel, 0), indicating that the photobleaching phenotype resulted from silencing of the endogenous *PDS* gene. *PDS*-related siRNA was detected in treated, bleached At-PDSi seedlings (Figure 3f, lower



**Figure 3.** Inducible *PDS* silencing in transgenic *Arabidopsis thaliana* plants (At-PDSi) at seed germination stage.

(a–d) At-PDSi seeds of line 2 were germinated on selective media in the absence (a) or presence of an inducer (b, c, d).

(b) Seedlings with induced *PDS* silencing showed a uniform photobleaching phenotype in the cotyledons at 6 days.

(c, d) *PDS* silencing was seen in the first pair of true leaves as well as the leaves that developed later (c, 9 days; d, 28 days). Scale bars = 0.5 cm in (a, b, d) and 0.1 cm in (c).

(e) Seeds of At-PDSi line 1 (a single transgenic locus) were germinated on MS in the presence of 17 $\beta$ -estradiol without the selective antibiotic hygromycin. Fifteen out of 19 plants showed induced *PDS* silencing. The photograph was taken at 29 days post-induction. Scale bar = 0.5 cm.

(f) Northern analysis of inducible *PDS* silencing in At-PDSi plants. Total high (top and middle panels) and low (lower panel) molecular weight RNAs were extracted from WT Columbia seedlings (0), uninduced At-PDSi control seedlings (-), and a mixture of induced At-PDSi seedlings, which showed a photobleaching phenotype (+). The blot (top panel) was probed with a *PDS*-specific sequence corresponding to the 3' region for detection of the endogenous *PDS* mRNA. Each lane contained 5  $\mu$ g RNA, and 28S RNA visualized by methylene blue staining was used as a loading control. Bottom panel shows the blot for siRNA, which was hybridized with a <sup>32</sup>P-labeled transcript corresponding to the 5'-terminal region (400 nt) of *PDS*. Each lane contained 50  $\mu$ g RNA. The arrow indicates the position of a 25-base DNA oligonucleotide.

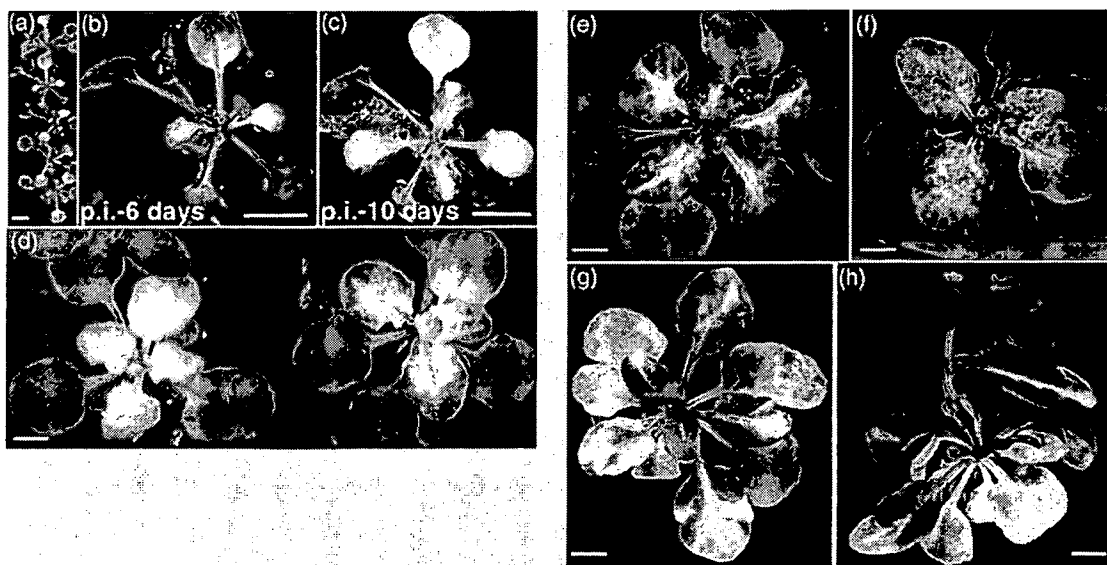
panel, +) using a *PDS*-specific probe, whereas no *PDS*-related siRNA could be detected in either WT control seedlings (Figure 3f, lower panel, 0) or untreated At-PDSi seedlings (Figure 3f, lower panel, -).

To rule out any toxic or non-specific secondary physiological effects because of 17 $\beta$ -estradiol-induced *PDS* silencing, T<sub>2</sub> seeds of At-PDSi line 1, which showed a 3 : 1 segregation ratio for Hyg<sup>R</sup>:Hyg<sup>S</sup>, were germinated on an inducer-containing medium without hygromycin, the selective antibiotic. After 3 weeks of incubation, 15 out of 19 T<sub>2</sub> seedlings showed the photobleaching phenotype and ceased to grow (Figure 3e), whereas the remaining four seedlings were normal. These four seedlings were sensitive to hygromycin as they ceased to grow after being transferred to a hygromycin-containing medium. The approximately 3 : 1 segregation pattern of both the selection marker and the photobleaching phenotype suggested that the four hygromycin-sensitive plants were WT, in agreement with the Mendelian segregation ratio for a single transgenic locus. These four plants showed no response to 17 $\beta$ -estradiol and did not exhibit any morphological alteration, indicating that the inducer had no secondary non-specific physiological effect on WT plants. Our results suggest that the inducible RNAi system is able to silence the endogenous *PDS* of *Arabidopsis* at the seed germination stage with comparable efficiency and effectiveness as the constitutive 35S-*PDSi* transgene.

We also examined post-germination induction of *PDS* silencing. Two- or four-week-old T<sub>2</sub> At-PDSi seedlings on the culture medium in the absence of the inducer were transferred to the inductive medium, and similar results were obtained from the seedlings of both age groups. We

observed two photobleaching phenotypes. In the first group which includes At-PDSi lines 2, 5, 7, and 8, a strong *PDS* silencing was seen, 1 week after induction, with newly emerged leaves showing uniform bleaching surrounding the central area of the leaves. The bleaching was subsequently propagated to the entire leaf. Figure 4(a–d) shows results from one representative line 2. Most of these plants with a strong photobleaching phenotype stopped growing. The second group includes At-PDSi lines 1, 3, 4, 6, and 9–12, and these lines showed varying photobleaching phenotypes. After 2 weeks of induction, photobleaching was limited to the areas near the veins (Figure 4e, a plant of line 12) or to the white/green patchy regions in the entire leaf (Figure 4f, a plant of line 1); however, the leaves became near-white over the next 2 weeks (Figure 4g,h, a plant of line 1). Although the *PDS* silencing extended to most rosette leaves and some cauline leaves (Figure 4h), these plants with varying degree of *PDS* silencing could still develop normally and were fertile after transfer to the soil.

We collected seeds from six T<sub>2</sub> plants of At-PDSi line 1 with induced silencing. When germinated on the selective medium (MS + Hyg), none of the T<sub>3</sub> progeny showed *PDS* silencing. When the seeds were germinated on MS medium with neither the selective antibiotic nor the inducer, four T<sub>2</sub> lines showed no *PDS* silencing. On the other hand, more than 10% of the progeny seedlings of the other two T<sub>2</sub> lines showed a constitutive photobleaching phenotype. The constitutive bleached plants presumably derived from some



**Figure 4.** Post-germination induction of *PDS* silencing in At-PDSi *Arabidopsis thaliana* transgenic plants.

(a–d) Strong induction of *PDS* silencing was seen in 2-week-old seedlings of line 2 after transfer to the inductive medium for 6 days (a, b) or 10 days (c). Note that (b) and (c) are from the same plant. Four-week-old seedlings of line 2 were transferred to the inductive medium and the picture was taken at 2 weeks post-induction (d).

(e–h) Delayed onset of *PDS* silencing. Four-week-old seedlings from different At-PDSi transgenic lines 12 (e) and 1 (f–h) were transferred to the inductive medium. Photobleaching was limited to the perivascular regions (e) or white/green patchy regions (f). The plants were photographed at 2 weeks after induction. Photobleaching progressed with time in the old leaves and extended to new rosette and cauline leaves when plants were photographed at 3 weeks (g) or 4 weeks (h) after induction. All scale bars represent 0.5 cm.

converted germ line cells in the L2 layers of  $T_2$  plants that have undergone  $17\beta$ -estradiol-induced *Cre/loxP* DNA recombination (Zuo et al., 2001), and therefore expressed G10-90-*PDSi* constitutively (Figure 1b).

#### Molecular analyses of inducible *PDS* silencing in *At-PDSi* plants

The delayed onset of *PDS* silencing in the second group prompted us to analyze the relationship between the photobleaching phenotype, endogenous *PDS* mRNA levels, and dsRNA induction upon  $17\beta$ -estradiol-induced *Cre/loxP* DNA excision.  $T_2$  progenies of *At-PDSi* line 1 were analyzed in detail. First, dsRNA corresponding to the *PDSi* transcript region (Figure 1) was analyzed. Two-week-old seedlings of line 1-1 (heterozygous) and line 1-2 (homozygous) were transferred to the inductive medium. RNA was extracted from a portion of the seedlings at 42 h post-induction. Total RNA was digested with *Rnase1*<sup>TM</sup> (Promega, USA), and dsRNA was analyzed by hybridization with a *PDSi* 5'-terminal probe containing sequences corresponding to the *PDSi* region (Figure 1). Figure 5(b) shows that signals of the expected size were detected in the treated seedlings of both lines 1-1 and 1-2 (lanes 2 and 3), but not in the untreated seedlings of line 1-1 (lane 1).

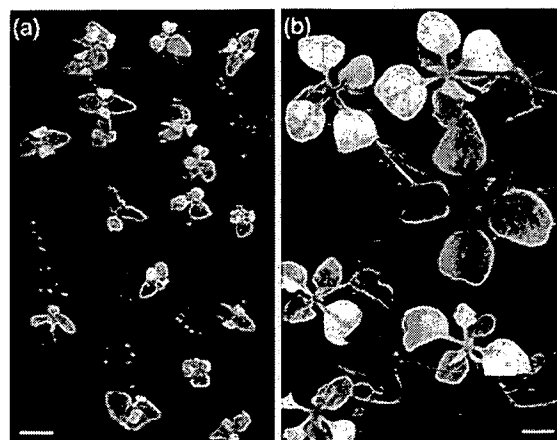
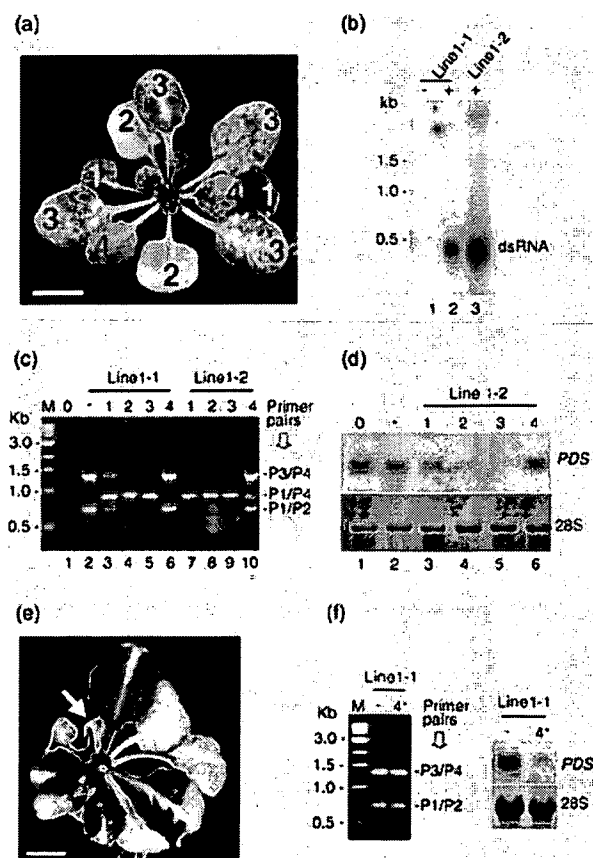


Figure 6. Inducible *PDS* silencing with uniform photobleaching phenotype in one *Nicotiana benthamiana* transgenic (Nb-*PDSi*) line.

(a) Seeds were germinated on the selective medium in the presence of the inducer (MS + Hyg +  $17\beta$ -estradiol), and seedlings were photographed at 2 weeks post-germination.

(b) Two-week-old seedlings were transferred to the inductive medium without hygromycin, and seedlings were photographed at 2 weeks post-induction. The green plant is presumably a WT plant that exhibited no secondary effect upon  $17\beta$ -estradiol treatment. Scale bar = 0.5 cm.

Figure 5. Molecular characterization of delayed onset of *PDS* silencing in *A. thaliana* transgenic plants.

(a) Two-week-old seedlings of *At-PDSi* line 1-1 were transferred to the inductive medium, and the plant was photographed at 18 days post-induction. Scale bar represents 0.5 cm. Leaves with increasing degrees of photobleaching were labeled with numbers 1–4, and those with a similar phenotype were labeled with the same number. Leaves designated 1 were formed before seedlings were transferred to the inductive medium.

(b) Seedlings of *At-PDSi* line 1-1 and 1-2 were either untreated (–) or treated (+) for 42 h with  $17\beta$ -estradiol, RNA was analyzed for the presence of pre-silencing dsRNA. Lane 1, line 1-1, untreated, 100  $\mu$ g RNA; lane 2, treated line 1-1, 50  $\mu$ g RNA; lane 3, treated line 1-2, 100  $\mu$ g RNA. RNAs were digested with *Rnase1*<sup>TM</sup> before being loaded onto formaldehyde-containing gels. The filter was hybridized with a *PDSi*-specific 5' sequence corresponding to the *PDSi* region (Figure 1a). Molecular weight standards are shown on the left.

(c) PCR analysis of genomic DNA prepared from WT Columbia (lane 1), untreated line 1-1 (lane 2), 18-day  $17\beta$ -estradiol-treated line 1-1 (lanes 3–6) or line 1-2 (lanes 7–10). Leaves with similar phenotype (panel a) were pooled from several plants, and the group numbers indicated on top of the blot. Primers P1–4 were used as illustrated in Figure 1(b). The expected PCR products from different combinations of primer pairs were indicated on the right, M, DNA molecular markers.

(d) Northern analysis of endogenous *PDS* mRNA levels. RNAs were extracted from the same samples as for DNA preparation (panel c), and the corresponding group numbers are indicated on the top. Each lane contained 5  $\mu$ g RNA.

(e) Showing one plant of *At-PDSi* line 1-1 treated with  $17\beta$ -estradiol for 18 days as described in (a) one week after been transferred to high light intensity. Note that the silenced leaves including the old ones (one indicated by arrow) became strongly bleached. Compare this plant to those shown in Figure 4(g,h). Scale bar = 0.5 cm.

(f) Molecular analysis of inducer-treated transgenic plants 1 week after inducer withdrawal and transfer to the soil. Some seedlings of *At-PDSi* line 1-1 treated with  $17\beta$ -estradiol for 18 days as described in (a) were transferred to the soil. After 1 week, leaves belonging to group 4 as well as two younger leaves that displayed photobleaching phenotype were pooled for PCR (left panel) and Northern blot analysis (right panel). Untreated line 1-1 was used as controls (–). For right panel, each leaf contained 5  $\mu$ g RNA.

For the remaining seedlings on the inductive medium, new leaves that emerged after 1-week induction also became patchy in appearance, and photobleaching progressed with time in both the heterozygous  $T_2$  line 1-1 and the homozygous  $T_2$  line 1-2, similar to that in the parental  $T_1$  line 1. To facilitate further analysis, leaves with varying degrees of photobleaching were numbered as shown in Figure 5(a).

PCR analysis was performed using primers specific for the excised sequences and flanking non-excised sequences (see Figure 1b). Based on a previous study (Zuo *et al.*, 2001), the P1/P2 and P3/P4 primer pairs were expected to yield PCR fragments of 696 and 1331 bp, respectively, from a non-recombinant T-DNA. By contrast, following Cre/*loxP*-mediated recombination and reconstitution of the G10-90-*PDSi* transcription unit, the P1/P4 primer pair produced a PCR product of 992 bp (Figure 1b). Leaves with similar phenotype were pooled from several plants for DNA preparation at 18 days post-induction. No fragment was amplified from WT Columbia (Figure 5c, lane 1), whereas P1/P2 and P3/P4 fragments were detected in untreated line 1-1 (Figure 5c, lane 2), indicating no DNA recombination. The P1/P4 fragment was detected in bleached (group 2 leaves) and patchy leaves (group 3 leaves) of both line 1-1 (Figure 5c, lanes 4 and 5) and line 1-2 (Figure 5c, lanes 8 and 9), indicating complete DNA excision in these leaves after 2 weeks of inducer treatment. In the near-green young leaves (group 4 leaves), however, varying amounts of the DNA excision were found (Figure 5c, lanes 6 and 10). There was no DNA excision in the sample collected from group 4 leaves of line 1-1 because only P1/P2 and P3/P4 fragments were amplified (Figure 5c, lane 6). By contrast, P1/P2, P3/P4, and P1/P4 fragments were detected in the samples collected from group 4 leaves of line 1-2 (Figure 5c, lane 10), indicating that DNA recombination occurred in some of the leaves.

We also found that DNA excision occurred in the old leaves (group 1 leaves) that were already developed before plants were transferred to the inductive medium. P1/P4 fragment was amplified from both line 1-1 (incomplete excision; Figure 5c, lane 3) and line 1-2 (complete excision; Figure 5c, lane 7), consistent with the highly effective inducer-dependent DNA recombination, probably as a result of penetration of 17 $\beta$ -estradiol to almost all cells in the lower leaves.

Endogenous *PDS* mRNA levels of  $T_2$  line 1-2 were assessed by Northern analysis 18 days after induction. Consistent with the photobleaching phenotype and DNA excision, *PDS* mRNA levels were significantly decreased in groups 2 and 3 leaves (Figure 5d, lanes 4 and 5), and slightly decreased in group 4 leaves compared to that in untreated line 1-1 control seedlings (lane 2) and WT Columbia control seedlings (lane 1). As expected, *PDS* mRNA was also degraded in group 1 old leaves (Figure 5d, lane 3),

consistent with the DNA excision assay. Although *PDS* silencing in old leaves was indeed induced after treatment, the persistence of the green color presumably resulted from the continued presence of carotenoids that were synthesized before the  $T_2$  seedlings were transferred to the inductive medium. This residual amount of pre-formed carotenoids was able to protect chlorophylls from photo-oxidation under low light intensity (our assay condition: 35  $\mu\text{mol sec}^{-1} \text{m}^{-2}$ ). However, when these plants were transferred to high light intensity (70  $\mu\text{mol sec}^{-1} \text{m}^{-2}$ ), all leaves including the old ones became strongly bleached (Figure 5e).

Some line 1-1 seedlings treated with the inducer for 18 days were transferred to the soil, and the phenotype of group 4 leaves as well as that of the newly emerged rosette leaves was followed. Group 4 leaves became patchy in appearance, and they became progressively bleached until near-white in appearance in 1 week. Similar results were observed in another two newly grown rosette leaves, which displayed limited areas of photobleaching. DNA and RNA were extracted from new photobleached leaves (including group 4 leaves), and PCR analysis showed that only P1/P2 and P3/P4 fragments were amplified (Figure 5f), indicating no DNA excision in these leaves. However, RNA analysis demonstrated a severe reduction in *PDS* mRNA levels, indicating that the *PDS* gene was silenced presumably via signals generated by the lower leaves.

#### *Inducible silencing of endogenous PDS gene in transgenic Nicotiana benthamiana*

Similar results were obtained in *N. benthamiana* transformed with the pX7-*PDSi*(Nb) construct. Inducible *PDS* silencing was observed when the inducer was given at germination (Figure 6a) or post-germination stages (Figure 6b). Northern analysis of endogenous *PDS* mRNA levels (data not shown) confirmed the visual photobleaching phenotype, suggesting that the inducible RNAi system is capable of inducing endogenous gene silencing in *N. benthamiana* and probably other plant species as well.

#### Discussion

In this work, we report the development of an inducible dsRNA-mediated silencing (inducible RNAi) system for conditional gene silencing in transgenic plants. This system contains two steps: (i) the inducible expression of dsRNA resulting from 17 $\beta$ -estradiol-induced DNA recombination and (ii) induction of target gene silencing by the dsRNA. A detailed characterization of the 17 $\beta$ -estradiol-inducible silencing system in transgenic *A. thaliana* and *N. benthamiana* plants demonstrated that this system is stringently controlled and can produce, at high efficiency, conditional silencing of both a *GFP* transgene and an

endogenous *PDS* gene and without any detectable secondary affect. At seed germination stage, all tested At-PDSi lines showed a uniform photobleaching phenotype similar to that obtained with constitutively silenced 35S-PDSi lines. This suggests that the efficiency and effectiveness of the inducible RNAi system against endogenous genes are comparable to those obtained with constitutive expression of dsRNA (constitutive RNAi).

The highly efficient gene silencing obtained by intron-containing dsRNA expressed from a constitutive promoter would produce loss-of-function transgenic plants similar to null mutants. If the target gene is essential for basic cell function and development, constitutive RNAi would probably prevent shoot regeneration, cause plant lethality or embryogenesis defect, and block the ability of the transformed plants to produce subsequent generations. These problems can be avoided by using the inducible RNAi system described here. Moreover, the inducible RNAi system provides the possibility to induce gene silencing at different stages of plant development post-germination. This is demonstrated by the At-PDSi lines described here, in which a portion of the tested 2- or 4-week-old seedlings showed strong photobleaching after induction similar to that shown by transgenic plants with 35S-PDSi phenotypes. Moreover, the varying initiation and degrees of inducible gene silencing generate plants with a range of loss-of-function phenotype much like an allelic series, facilitating functional analysis.

Both heterozygous (line 1-1) and homozygous (line 1-2) progeny of At-PDSi line 1 retain the ability to reproduce inducible *PDS* silencing as the parental line, indicating that the inducible RNAi can be transmitted to the next generation. Having stable and reproducible RNAi transgenic lines would allow genetic crosses to be made and investigations of gene functions to be carried out with subsequent generations. Other silencing induction systems, such as *Agrobacterium* infiltration (Voinnet and Baulcombe, 1997) and infection with vectors derived from RNA viruses (Dalmay et al., 2000; Ratcliff et al., 2001) or DNA viruses (Peele et al., 2001; Turnage et al., 2002), have limited utility for functional genomics because of their transient nature, and the silencing effects are not heritable. Moreover, *Agrobacterium* infiltration cannot be applied to *Arabidopsis* because of its small plant size and clumpy rosette leaves. In the case of virus-induced gene silencing, some infected plants display stunted growth and they often do not produce inflorescences, flowers, or seeds (Turnage et al., 2002). Moreover, disease symptoms caused by viral infection could confound the interpretation of the phenotype as a result of silencing of the target gene. By contrast, the inducible RNAi system described here can be triggered by simply treating plants with the inducer for a certain period of time. Treated plants display a specific silencing phenotype without any non-specific effects (see Figures 2a, 4e–h and 6b).

We have chosen the inducible Cre/loxP DNA excision system (CLX) rather than the XVE transient inducible system (Zuo et al., 2000) to produce the inducible RNAi for several reasons. The mechanism for RNA silencing involves an initial induction process followed by a systemic spread of the silencing signal (for review, see Mlotshwa et al., 2002). The local initiation of gene silencing by dsRNA is equally effective for both transgene and endogenous genes (Smith et al., 2000; Wesley et al., 2001). However, unlike the widespread, persistent silencing observed for a *GFP* transgene, systemic silencing of endogenous genes was transient and limited (Palauqui and Vaucheret, 1998; Voinnet et al., 2000). Amplification of the signal is necessary for efficient systemic silencing (Palauqui and Vaucheret, 1998; Voinnet et al., 1998). It has been proposed that the silencing signal is perpetuated by transgenes, but not by endogenous genes (Fagard and Vaucheret, 2000; Mlotshwa et al., 2002). The CLX system can rescue the weak amplification of the silencing signal by endogenous genes. Once the Cre/loxP-mediated DNA excision occurred upon inducer treatment, expression of the downstream intron-containing dsRNA would be permanently activated. This situation mimics the expression of an RNAi using a constitutive promoter, which is the most efficient, effective, and high-throughput system for gene silencing, and the systemic spread of silencing signal is therefore not required. Expression of dsRNA from the inducible XVE system (Zuo et al., 2000) without DNA recombination would require repeated applications of 17 $\beta$ -estradiol to the transgenic plants for sustained expression, and this may not be practical for plants growing in the soil.

The strong photobleaching induction in At-PDSi lines (lines 2, 5, 7, and 8) at post-germination stages is believed to be a result of complete DNA excision, which would reconstitute the G10-90-*PDSi* transcription unit to produce *PDSi* transcripts constitutively throughout the entire plant. In this case, no silencing signal amplification was required. This interpretation is consistent with our PCR analysis of At-PDSi lines (second group), displaying a delayed onset of gene silencing. In both line 1-1 and line 1-2 leaves, complete excision of DNA within the loxP sites showed strong photobleaching or patchy *PDS*-silencing phenotype. In near-green leaves where incomplete or no DNA excision was detected, only a weak systemic spread (weak signal amplification) of *PDS* silencing to upper rosette leaves was seen during the 18-day incubation with the inducer (Figure 5a–d). However, upon an additional week of growth after inducer withdrawal, strong *PDS* gene silencing was also detected in group 4 leaves and in the next two younger leaves (Figure 5f). These results suggest a limited systemic translocation of the silencing signal to two to three upper leaves.

Because of 17 $\beta$ -estradiol instability (Zuo et al., 2000), a second or even multiple treatment with fresh inducer may

be needed to fully reactivate this RNAi system in some transgenic lines. With appropriate improvement of the induction conditions, a higher DNA excision efficiency, and therefore a higher proportion of lines showing strong induction, may be obtained. Nevertheless, the incomplete *Cre/loxP* DNA excision, which results in genetic chimera in transgenic plants, may provide a useful system to study mechanisms of long-distance signal transduction in gene silencing in *Arabidopsis*, which is difficult to graft (Turnbull *et al.*, 2002). The mechanisms involved in systemic RNA silencing in plant systems are being actively investigated using grafting and transient expression approaches with *N. benthamiana* or *N. tabacum* (Guo and Ding, 2002; Mallory *et al.*, 2001; Voinnet *et al.*, 2000). No mutations specific to systemic silencing have yet been reported in plant systems. Because of the small plant size and the clumpy rosette leaves, it is impossible to carry out localized infiltration of *Arabidopsis* with *Agrobacterium*, and grafting manipulation in *Arabidopsis* is also a challenging task. For these reasons, the ability to generate genetic chimera in transgenic *Arabidopsis* producing RNAi only from treated tissues would be very useful for future investigations. As shown by PCR analysis in At-PDSi line 1-1 and line 1-2, DNA excision occurred in lower rosette leaves, but not in upper rosette leaves. Gene silencing resulting from local RNAi induction (complete excision, Figure 5c,d) or signal-mediated long-distance (no excision) induction (Figure 5f) can be predicted by simple PCR analysis. The ability to generate genetic chimera in *Arabidopsis* may also find useful applications in research on other types of long-distance signaling (e.g. flowering time) in plants.

## Experimental procedures

### Plasmid construction

DNA manipulations and cloning were carried out using standard procedures (Sambrook *et al.*, 1989). The third intron of *Arabidopsis* actin gene 11 (ATU27981, nt 1957–2111) was selected for the intron-containing intermediate construct (pSK-int). This intron was amplified by PCR using two primers: Pint5', 5'-TACGTAAGTATGATCTTCAACACC-3'; and Pint3', 5'-GGAATTCTGCAACACACAGACAAT-3'. The primers were designed such that their border sequences contained the consensus sequence (bold letters) for plant introns: AG//GTAAGT...TGCAG//G (Shapiro and Senapathy, 1987). Two restriction sites *Sna*BI and *Eco*RI (underlined) were added for cloning purposes. A PCR fragment of 155 bp was digested with *Sna*BI/*Eco*RI and cloned into *Eco*RV/*Eco*RI-digested pBluscript II SK+ to yield the intermediate construct pSK-int (Figure 1a).

To clone sequences encoding the inverted-repeat RNA into the pSK-int intermediate vector, a 357 bp fragment corresponding to nucleotides (nt) 360–716 of the *GFP* 3'-terminal sequence (Voinnet and Baulcombe, 1997) was cloned into the 5' and 3' arms of the intron (Figure 1a), and the resulting plasmid was named as pSK-GFPi. The 5'-terminal sequences of *PDS* of *A. thaliana* and *N. benthamiana* were obtained by RT-PCR amplification with

specific primers. For *A. thaliana* *PDS*, the primers were: Pat5', 5'-GACTAGTATGGTTGTGTTGGGAATG-3'; and Pat3', 5'-GATATCCTTCATGCAGCTATC-3'. These primers were used to obtain a fragment of 405 bp corresponding to nt 128–532 of the *A. thaliana* *PDS* cDNA (L16237), and *Spe*I and *Eco*RV restriction sites (underlined) were added to the cDNA fragment. For PCR amplification of the *N. benthamiana* *PDS* sequence, the primers were: Pnb5', 5'-GACTAGTATGCCTCAAATTGGACTTGT-3'; and Pnb3', 5'-CAGCTG-TAGACAAACCACCAAAC-3' homologous to regions of the tomato *PDS* cDNA (M88683) nt 318–337 and nt 676–696, respectively. These primers were designed with the addition of *Spe*I and *Pvu*II restriction sites (underlined). Using *N. benthamiana* RNA as templates, a 386 bp fragment was obtained with RT-PCR, whose sequence exhibited high homology with the tomato *PDS* cDNA. RT-PCR fragments derived from *A. thaliana* (At) and *N. benthamiana* (Nb) were cloned into the pCR-Blunt vector (Invitrogen, USA) to give pCR-PDS(At) and pCR-PDS(Nb), respectively. Fragments of *Spe*I-*Eco*RI and *Hind*III-*Xho*I were inserted into both arms of the intron of pSK-int digested with the appropriate restriction enzymes as shown in Figure 1(a) to obtain pSK-PDSi(At) and pSK-PDSi(Nb), respectively.

For inducible dsRNA transformation constructs, the kanamycin-resistance gene in pX6-GFP (Zuo *et al.*, 2001) was replaced with a hygromycin-resistance gene, and the derivative called pX7-GFP. To create an inducible expression of intron-containing dsRNA, fragments of *Xho*I-*Xba*I from pSK-GFPi, pSK-PDSi(At), and pSK-PDSi(Nb) were subcloned into pX7-GFP digested with *Xho*I/*Spe*I (*Xba*I and *Spe*I are compatible), resulting in pX7-GFPi, pX7-PDSi(At), and pX7-PDSi(Nb), respectively (Figure 1b).

In addition, the *Pst*I-*Sac*I fragment from pSK-PDSi(At) was cloned into a modified binary vector pCAMBIA-1300 (AF234296), which contained a 35S promoter and a 35S terminator, to give pCAMBIA-PDSi(At), which is a constitutive RNAi construct (Figure 1c).

Upon request, vectors described in this paper are available to academic researchers for non-commercial projects.

### Plant materials, transformation, and growth conditions

A transgenic *N. benthamiana* line (GFP-16c) carrying a 35S-GFP transgene with a kanamycin-selectable marker at a single locus in homozygous condition (Ruiz *et al.*, 1998) and a transgenic *A. thaliana* ecotype C24 line (Dalmay *et al.*, 2000) carrying a similar transgene were used for pX7-GFPi transformation. *A. thaliana* ecotype Columbia was used for pX7-PDSi(At) and pCAMBIA-PDSi(At) transformation. WT *N. benthamiana* was used for pX7-PDSi(Nb) transformation. *N. benthamiana* transformation was carried out by co-culture with *Agrobacterium*, whereas *A. thaliana* was transformed by the floral dip method (Clough and Bent, 1998). The selective medium contained MS medium plus hygromycin (20 mg l<sup>-1</sup> for *A. thaliana* and 40 mg l<sup>-1</sup> for *N. benthamiana*), whereas the inductive medium contained, in addition, 17 $\beta$ -estradiol (2  $\mu$ M). GFP fluorescence was examined using a 100 W hand-held long-wavelength UV lamp.

### Analyses of RNA and DNA

Total RNA was isolated from plant tissues by LiCl precipitation (Verwoerd *et al.*, 1989). The LiCl supernatant fraction was precipitated with 3 volumes of ethanol to obtain genomic DNA and low molecular weight RNA (siRNA). dsRNA was obtained by digesting total RNA with Rnase1<sup>TM</sup> (Promega, USA) (0.5 U Rnase1  $\mu$ g<sup>-1</sup> total RNA) at 37°C for 3 h. For Northern analysis, total RNA or dsRNA was separated on 1.2% agarose formaldehyde gels, transferred to

Hybond-N+ membranes, and hybridized with  $^{32}\text{P}$ -labeled cDNA probes specific for the respective RNA. Low molecular weight RNA analysis was done as described (Hamilton and Baulcombe, 1999; Llave et al., 2000). The probes for GFP and PDS siRNA were  $^{32}\text{P}$ -labeled 3'-terminal 356 nt of GFP or 5'-terminal 400 nt of PDS antisense RNA, respectively, transcribed by T7 RNA polymerase. The PCR analysis with approximately 200 ng of genomic DNA was subjected to 94°C for 20 sec, 50°C for 20 sec, and 72°C for 2 min for 30 cycles. Primers for PCR analysis were: P1, 5'-GCCGCCACG-TGCCGCCAGTGCCGCC-3'; P2, 5'-CTCGTCAATTCCAAGGGCAT-CGGT-3'; P3, 5'-CTGGACACAGTGCCCGTGTCCGA-3'; P4, identical to Pint3' for intron amplification (see Results).

### Acknowledgements

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## TECHNICAL ADVANCE

# Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA

Shuai Chen, Daniel Hofius, Uwe Sonnewald and Frederik Börnke\*

Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Corrensstrasse 3, 06466 Gatersleben, Germany

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\*For correspondence (fax +49 39482 5515; e-mail boernke@ipk-gatersleben.de).

## Summary

Downregulation of endogenous genes via post-transcriptional gene silencing (PTGS) is a key to the characterization of gene function in plants. The recent discovery that double-stranded RNA (dsRNA) is an extremely effective trigger of gene silencing greatly enhanced the predictability of this approach. However, strong constitutive silencing often leads to pleiotropic effects, which make it difficult to directly relate phenotype to gene function, or even interferes with the recovery of viable transgenic plants. Here, we show that strong genetic interference can be achieved in a chemically inducible fashion, allowing for temporal and spatial control of gene silencing in transgenic plants. To this end, transgenic tobacco plants were established expressing dsRNA in the form of intron-spliced hairpin structures under the control of the ethanol-inducible *alc* gene expression system. Targeting magnesium (Mg)-chelataase subunit I (*Chl I*) and glutamate 1-semialdehyde aminotransferase (*GSA*), both involved in chlorophyll (*chl*) biosynthesis, resulted in rapid and specific mRNA degradation upon induction with ethanol. Ethanol-inducible silencing of the target genes caused strong but transient phenotypical alterations featured by a progressive loss of *chl* in young leaves, which persisted for about 7–9 days before newly growing leaves completely recovered. About 10–30% of the primary transformants showed phenotype development upon induction. Local silencing of *Chl I* could be achieved by confined ethanol treatment of a single leaf without affecting any other part of the plant. Inducible gene silencing using the *alc* system promises to obviate the problems associated with constitutive RNA silencing and enables to dissect primary and secondary effects of PTGS at temporal and spatial resolution.

**Keywords:** chemically inducible expression, ethanol, double-stranded RNA, gene silencing, functional genomics, *Nicotiana tabacum*.

## Introduction

The recent completion of the *Arabidopsis thaliana* genome sequence (The Arabidopsis Genome Initiative, 2000) and the accumulation of sequence data from a number of other model plants and important crop species, such as rice (Goff *et al.*, 2002; Yu *et al.*, 2002), have provided a vast new resource to define gene function at the morphological, biochemical, or physiological level. A critical step in exploiting these genomic resources, however, depends on the development of novel tools and approaches for the functional analysis of a given gene. In the past, the use of loss-of-function or reduced-expression mutants has proven to be a powerful tool to relate a mutant phenotype to the function of a particular gene. Traditionally, these reverse

genetic approaches rely on transposon insertions or classic genetic screens (for review, see Page and Grossniklaus, 2002). However, their use is limited by the untargeted nature of the mutagenesis and their restriction to a few genetically tractable plant species. The discovery of the antisense phenomenon of plant gene silencing (van der Krol *et al.*, 1988; Smith *et al.*, 1988), and subsequently co-suppression (van der Krol *et al.*, 1990; Napoli *et al.*, 1990), provided an alternative means for investigating the role of specific gene products in plant growth and metabolism and has been particularly versatile in studying plant primary metabolism (Frommer and Sonnewald, 1995; Stitt and Sonnewald, 1995). The antisense and co-suppression

phenomena are collectively referred to as post-transcriptional gene silencing (PTGS), which describes a nucleotide sequence-specific RNA degradation process naturally providing a defense mechanism against invasive nucleic acids such as viruses, transposons, and transgenes (Baulcombe, 2002; Matzke *et al.*, 2001; Vance and Vaucheret, 2001; Voinnet *et al.*, 1998). A set of mechanistically related pathways were also found in fungi (quelling) and in animals (RNA interference (RNAi)) and requires a conserved set of gene products (for recent reviews, see Carthew, 2001; Cogoni and Macino, 2000; Plasterk, 2002). According to current models of PTGS, an endogenous RNA-dependent RNA polymerase initially synthesizes a double-stranded RNA (dsRNA) molecule using the target transcript as template (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000), which is subsequently processed into 21–25 ntRNA fragments of both polarities (Hamilton and Baulcombe, 1999). These short interfering RNAs (siRNAs) are then incorporated into a dsRNA-induced silencing complex (RISC) to guide cycles of specific RNA degradation (Hammond *et al.*, 2000; Tang *et al.*, 2003; Zamore *et al.*, 2000). In some experiments, however, when conventional antisense and co-suppression constructs are used to trigger PTGS, RNA-silencing occurs only in a portion of the transformants or their progenies or the constructs even fail to induce silencing, rendering these approaches largely ineffective. Recently, considerable progress in effectively triggering PTGS has been made by using constructs designed to express dsRNA fragments, usually in the form of self-complementary hairpin RNA, consistently yielding a high degree and frequency of PTGS. This often gives rise to phenotypes that resemble those of the null alleles of the target genes (Chuang and Meyerowitz, 2000; Levin *et al.*, 2000; Smith *et al.*, 2000). However, because of the high degree of silencing, which is achieved by this method, functional analysis of a particular gene product required during plant transformation and regeneration from culture is limited, as viable plants might not be recovered. Moreover, constitutive gene silencing consistently entails pleiotropic effects, which might superimpose the primary impact of reduced gene expression and thus mask true gene function. This is especially true, if metabolic processes are under scrutiny, as the observed phenotype could rather reflect a physiological adaptation to the loss of a metabolic function than a primary response to the latter. To overcome these problems a system for inducible gene silencing of candidate genes would be highly desirable.

The problems have partially been solved by the introduction of virus-induced gene silencing (VIGS) systems where a recombinant virus carrying a partial sequence of a host gene is used to infect the plant. When the virus spreads systemically, the endogenous transcripts, which are homologous to the insert in the viral vector, are degraded by PTGS (Baulcombe, 1999). Although several VIGS vectors have been described (Gosselé *et al.*, 2002; Holzberg *et al.*,

2002; Liu *et al.*, 2002; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002), these approaches suffer from a number of shortcomings. First, the use of each system is restricted by the host range of the virus it is derived from and thus no such system is broadly applicable to a wide range of plant species. Second, the VIGS phenotype is superimposed, and sometimes complicated, by at least mild disease symptoms of virus infection, which cause significant biochemical perturbations not directly linked to the suppression of the target gene. This causes problems especially in physiological investigations. Most of these limitations should be overcome by the use of a chemically inducible promoter to drive expression of the silencing construct, allowing the investigator to control when a specific gene will be inactivated. The optimal system would employ a non-toxic inducer with high specificity and minimal potential to elicit physiological responses. Moreover, the system should have the capacity to achieve high level of expression but with a concomitant negligible activity in the absence of the inducer. One such system is the *alc* gene switch based on a regulon derived from the filamentous fungus *Aspergillus nidulans* (Caddick *et al.*, 1998; Roslan *et al.*, 2001; Salter *et al.*, 1998). In plants, the system basically consists of two modules: the AlcR transcriptional regulator expressed from the cauliflower mosaic virus (CaMV) 35S promoter and a modified *alcA* promoter in front of the gene of interest. In the presence of ethanol, AlcR binds to the modified *alcA* promoter and drives expression of the target gene. Several studies demonstrated the efficiency of the *alc* system in a wide range of plant hosts, including *Arabidopsis*, *Brassica napus*, *Nicotiana tabacum*, and potato tubers (Caddick *et al.*, 1998; Junker *et al.*, 2003; Roslan *et al.*, 2001; Salter *et al.*, 1998; Sweetman *et al.*, 2002). Induction of the system can be achieved by either root drenching of the plants with ethanol solution (Caddick *et al.*, 1998; Roslan *et al.*, 2001; Salter *et al.*, 1998) or exposing the plants to ethanol vapor (Sweetman *et al.*, 2002). Alternatively, the *alc* gene switch can also be activated by other related chemicals. A recent study demonstrated a more rapid induction of the system upon the application of acetaldehyde, the physiological inducer of the *alc* regulon in *A. nidulans*, than that of ethanol (Junker *et al.*, 2003).

We initiated the study described herein to determine whether the *alc* system can be used to achieve inducible gene silencing in transgenic tobacco. To this end, nuclear genes involved in the chlorophyll (chl) biosynthetic pathway were targeted, because it was assumed that the host gene silencing would be easy to visualize and quantify as loss of chl. Our data demonstrate that transgenic tobacco plants designed to express double-stranded hairpin constructs under the control of the ethanol responsive promoter exhibit a rapid but transient development of the characteristic phenotype upon induction. Using repeated application of ethanol, extended periods of gene silencing

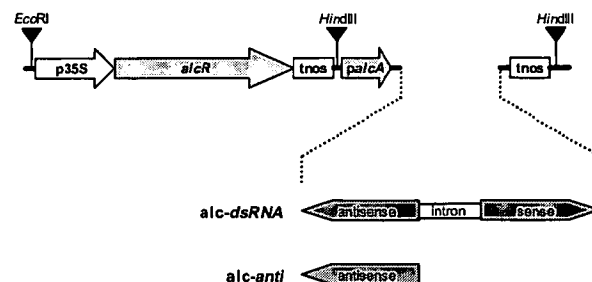
could be maintained. We further show that spatial silencing of a target gene could be achieved by ethanol treatment of a single tobacco leaf. With these attributes, inducible PTGS using the *alc* system promises to extend the kind of silencing studies that can be carried out in transgenic plants, in particular, with respect to temporal and spatial resolution of silencing effects, rendering the system extremely useful for metabolic studies.

## Results

### Construction of silencing vectors and plant transformation

To evaluate the suitability of the *alc* system for inducible gene silencing, two nuclear target genes were selected, which have previously been described to yield a readily discernable phenotype in conventional antisense experiments. Magnesium (Mg)-chelatase is a heteromeric enzyme complex composed of three-subunits (designated CHL I, CHL H, and CHL D) that catalyzes the incorporation of  $Mg^{2+}$  into protophorphyrin IX, which represents the first committed step in chl biosynthesis. Antisense suppression of *Chl I* in transgenic tobacco led to a strongly reduced green pigmentation as a result of the decreased chl biosynthetic capacity (Papenbrock *et al.*, 2000). A key regulatory step in tetrapyrrole biosynthesis in higher plants, providing the precursors for chl and heme synthesis, is the formation of 5-aminolevulinate catalyzed by the activity of glutamate 1-semialdehyde aminotransferase (GSA). Expression of GSA antisense RNA in tobacco plants results in a decline in chl content apparently leading to pale leaves (Höfgen *et al.*, 1994).

Constructs for inducible expression of dsRNA in transgenic plants were assembled in the appropriate plant transformation vector (Caddick *et al.*, 1998), which contained the *AlcR* gene driven by the constitutive CaMV 35S promoter and gene-specific fragments in sense and antisense orientation interspersed by a short intron under control of the modified *alcA* promoter (*alc-dsRNA* gene cassette, Figure 1). In parallel, inducible antisense constructs were made to target *Chl I* and GSA, respectively, by putting the corresponding antisense fragment under the control of the ethanol-inducible promoter (*alc-anti* gene cassette, Figure 1). After *Agrobacterium*-mediated gene transfer (Rosahl *et al.*, 1987), 80 primary transformants for each construct were transferred to the greenhouse. Prior to the application of ethanol, all transgenic plants were indistinguishable from wild-type plants, indicating that the promoter was not leaky. Northern analysis of transgene-specific dsRNA further confirmed tight control of the promoter under un-induced conditions in that no detectable levels of dsRNA were present prior to the application of the



**Figure 1.** Diagrammatic representation (not to scale) of *alc*-system-derived silencing constructs.

The *alcR* cDNA is under control of the CaMV 35S (*p35S*) promoter and positioned upstream of the *nos* terminator (*tnos*). The *alc-dsRNA* construct contains either a *Chl I* or GSA fragment in antisense and sense orientation separated by intron 1 of potato GA20 oxidase (200 bp) in spliceable orientation. The *alc-antisense* construct comprises simply the respective antisense fragment. In both constructs, all fragments were inserted downstream of the chimeric *palCA* promoter, which consists of the CaMV 35S minimal promoter (−31 to +5) fused at the TATA-box to upstream promoter sequences of *alcA* (Caddick *et al.*, 1998).

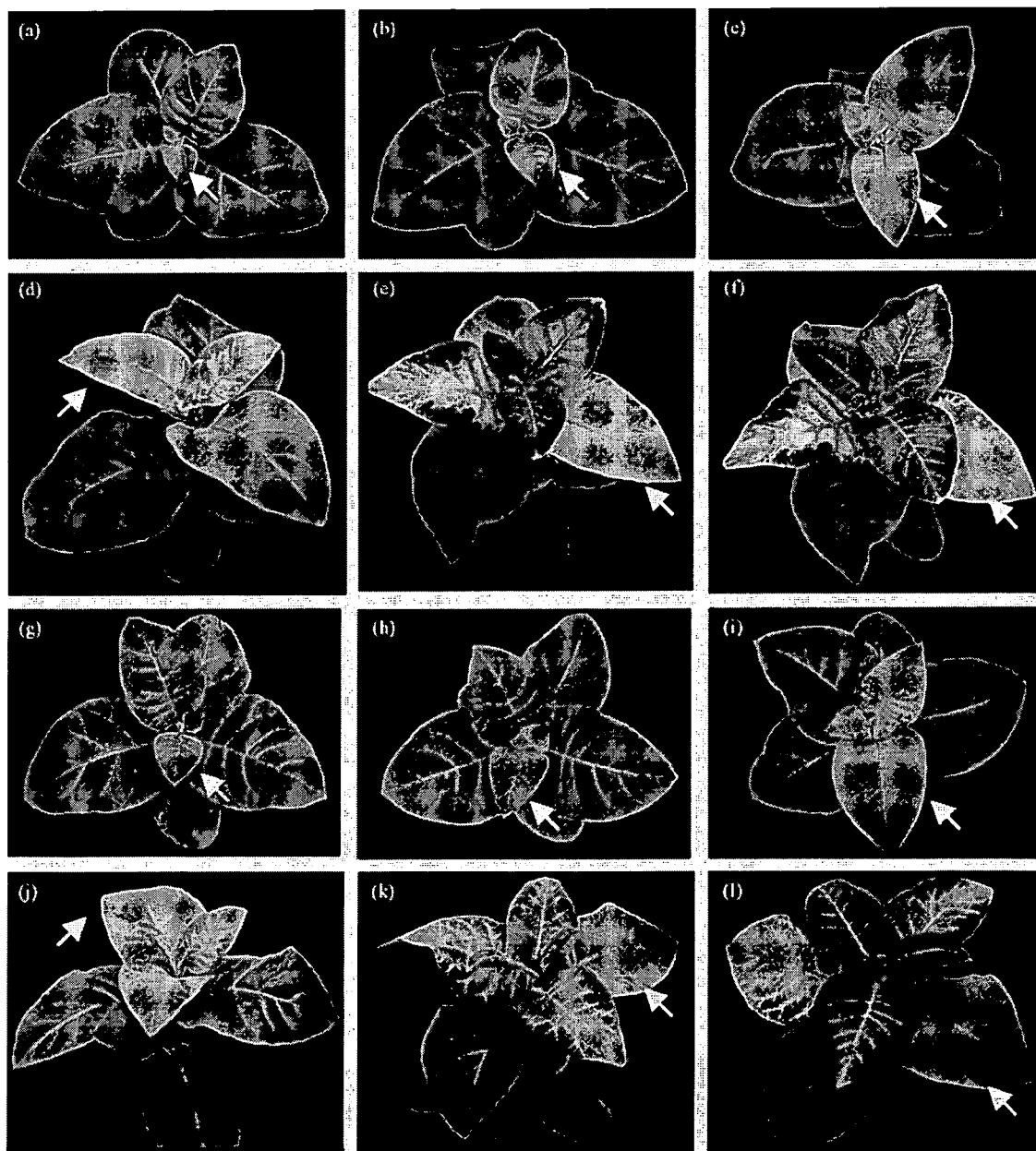
inducer (data not shown). However, after ethanol induction, 26 (33% efficiency) of the *alc-dsChl I* transgenics and 10 (13% efficiency) of the *alc-dsGSA* plants displayed the characteristic phenotype previously described from constitutively silenced plants. Phenotypes of individual transformants varied from heavily bleached leaves with only small green areas remaining (11 *alc-dsChl I* and 4 *alc-dsGSA* plants, respectively) to variegation patterns of likewise green and yellow to white patches (six *alc-dsChl I* and three *alc-dsGSA* plants, respectively) and pale areas along the major leaf veins (nine *alc-dsChl I* and three *alc-dsGSA* plants, respectively). The overall efficiency using the *alc* system for inducible gene silencing was much lower than that of the recently published inducible RNAi system based on estradiol induction (Guo *et al.*, 2003). Whether this was because of differences in constructs or vectors or because of a species difference is currently unknown.

Specific silencing of the target gene in phenotypically affected plants was further confirmed by Northern blotting using gene-specific probes (data not shown). Reduction in *Chl I* and GSA mRNA steady state level correlated with the strength of the observed phenotype. Contrastingly, none of the plants solely expressing the antisense fragment targeted against *Chl I* and GSA displayed any visible phenotype upon induction (data not shown). Northern blotting was applied to screen for individuals expressing the respective antisense transgenes. Several plants expressing considerable amounts of the antisense transcript could be identified for each construct (data not shown). From these data, we conclude that ethanol-inducible expression of dsRNA causes silencing of endogenous genes comparable to that in constitutively silenced plants. Selected primary transformants for each construct were selfed and the T1 generation was subject to a detailed analysis.

**Rapid and reversible phenotypical changes in plants containing *alc-dsRNA* expression cassettes**

Three *alc-dsChl I* lines (14, 16, and 45), and two *alc-dsGSA* lines (60 and 70) were chosen for a detailed analysis according to their strong inducible phenotype observed in the  $T_0$  generation. Seeds were germinated on kanamycin-containing medium and resistant seedlings were analyzed 4 weeks

after transfer to soil. Application of 1% ethanol by root drenching led to the development of the characteristic phenotype featured by the loss of chl. The phenotype started to develop approximately 36 h post-induction (hpi) for *alc-dsChl I* lines and approximately 48 hpi for *alc-dsGSA* lines, and was first visible in the top leaves (Figure 2). The bleached patches expanded as the leaves grew, and also appeared in nascent leaves over a period of



**Figure 2.** Time course of phenotype development in *alc-dsRNA* lines after application of ethanol.

(a–f) *alc-dsChl I* line 16 before, and 2, 4, 5, 9, and 12 dpi, respectively.

(g–l) *alc-dsGSA* line 60, before, and 2, 4, 5, 7, and 9 dpi. The arrow indicates the type of leaf that has been followed for molecular analyses.

Plants (42 days old) were induced via root drenching with 100 ml 1% (v/v) ethanol and photographs were taken at different time points.

7–9 days. After that, newly emerging leaves looked like the wild type again while those initially affected never recovered. Whether this was because of the developmental control of chl-synthesizing enzymes (Härtel *et al.*, 1997; He *et al.*, 1994) or because of stable silencing is currently unknown. Leaves that were mature prior to induction always remained green. Upon a second induction, the silencing phenotype could be re-established in the newly grown leaves of transgenic plants (data not shown).

In some cases, the silencing effect caused by a single induction might be too transient to reveal the full sequence of consequences of reduced gene expression. In order to investigate whether extended periods of gene silencing could be achieved by repeated ethanol treatments, *alc-dsChl I* plants were root-drenched with 1% ethanol every 2 days for 15 days in total. Phenotypic changes became apparent 2 days post-induction (dpi) but, in contrast to the single-induction experiments, they were maintained over the entire period of ethanol treatment (Figure 6b). Therefore, using re-iterated treatments, extended periods of gene silencing can be maintained.

#### Kinetics of inducible gene silencing

In order to follow the kinetics of target transcript reduction in *alc-dsRNA* lines, samples were taken from leaves expected to become phenotypically affected at different time points after application of ethanol. To compensate for plant-to-plant variation, at least three plants were probed at each time point and samples were pooled after preparation of total RNA. Northern analysis revealed that the *Chl I* mRNA was significantly reduced as early as 8 h after ethanol induction. Transcript levels further declined to undetectable levels at 48 hpi so as to rise again from 72 hpi onwards. The initial amount was reached again at approximately 9 dpi in newly emerging leaves, which were phenotypically normal (Figure 3a). *Chl I* transcript levels in ethanol-treated wild-type plants remained stable over the time course of the experiment (data not shown). Similar kinetics of target transcript decay and duration of mRNA downregulation was observed in the *alc-dsGSA* plants (Figure 3b). In the line under investigation (line 60), an additional band migrating above the endogenous GSA transcript appeared upon induction and was detectable upto 48 hpi. As PTGS has been shown to affect RNA processing (Mishra and Hanada, 1998), we used RT-PCR to investigate whether splicing of the endogenous GSA messenger was impaired in these plants. No PCR product that could possibly represent an incompletely spliced RNA was detected. However, if primers specific for the intron included in the transgene construct were used, a weak but specific band was amplified (data not shown). Therefore, the additional band on the GSA Northern blot was tenta-

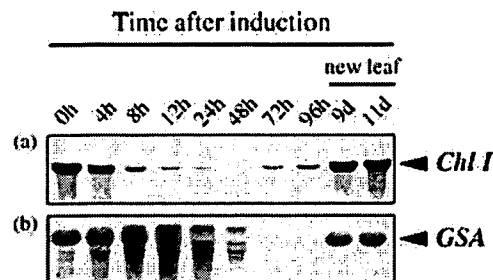


Figure 3. Efficient downregulation of target mRNA following ethanol-inducible expression of dsRNA constructs.

(a) Time course of *Chl I* transcript degradation in *alc-dsChl I* plants (line 16). (b) Time course of *GSA* mRNA degradation in *alc-dsGSA* plants (line 60). Transgenic tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being c. 5 cm in size at the time point of induction. After the indicated periods of time, total RNA was isolated and Northern blot hybridization was performed with the respective cDNA probe. At 9 and at 11 dpi, newly grown leaves were taken for the analysis.

tively assigned to the unspliced intron-containing inverted-repeat fragment. This band was never observed in any other *alc-dsGSA* line; however, line 60 had the strongest phenotype and high expression of the transgene might interfere with its correct splicing because of sub-optimal splice sites.

In order to follow the silencing process in *alc-dsGSA* lines on the protein level, samples were taken from the same leaves as before and subjected to a Western analysis using GSA-specific antibodies (kindly provided by Dr B. Grimm, Humboldt University, Berlin, Germany). Within 48 hpi, a considerable reduction of the GSA amount occurred in the transgenics (Figure 4), which is in good accordance with phenotype development in these plants. No detectable protein was observed 72–96 hpi. When newly developing leaves were probed for GSA 9 dpi, the amounts were comparable to those before induction.

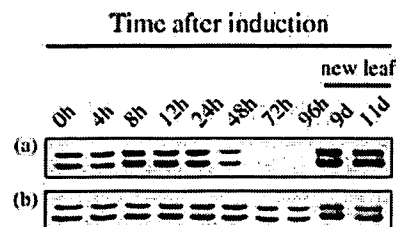
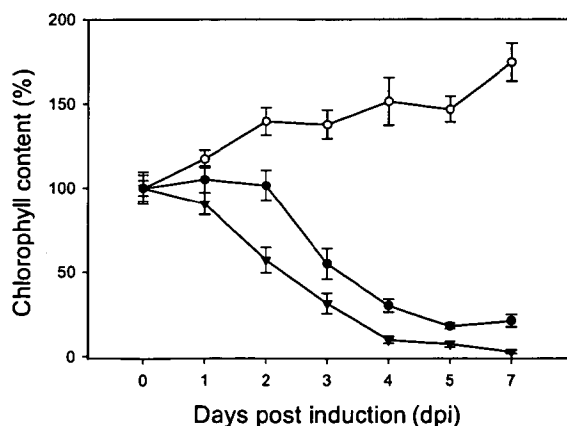


Figure 4. Time course of GSA protein degradation in *alc-dsGSA* plants after ethanol treatment.

(a) Level of GSA protein in *alc-dsGSA* plants (line 60) after induction. (b) Ethanol-treated control plants. Plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being c. 5 cm in size at the time point of induction. After the indicated periods of time, total protein was prepared and Western blot analysis was performed using an anti-GSA antibody. At 9 and at 11 dpi, newly grown leaves were taken for the analysis.

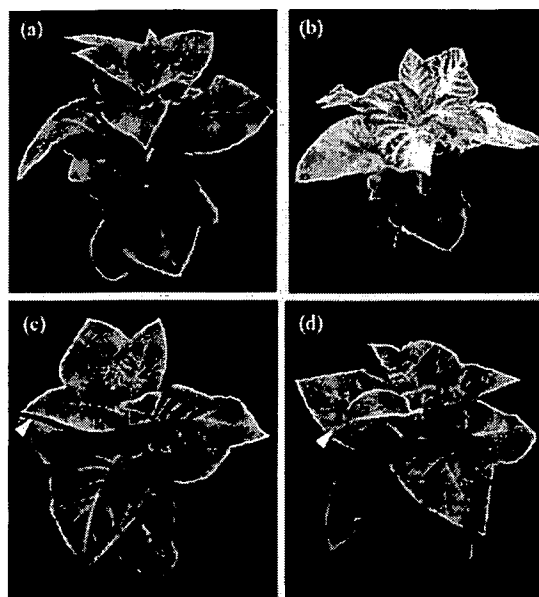
### Changes in chlorophyll contents upon induction of gene silencing

In order to investigate the degree of gene silencing, which could be achieved with the ethanol-inducible system compared to plants constitutively silenced for GSA and *Chl I* (Höfgen *et al.*, 1994; Papenbrock *et al.*, 2000), the decrease in chl content in phenotypically affected leaves over time was taken as an indirect measure for chl biosynthetic capacity. Therefore, leaves were followed that were approximately 5 cm in length at the time point of induction. Samples were taken at different time points and analyzed for their chl content. To compensate for local variations within a plant, two samples were taken from each leaf. To compensate for plant-to-plant variation, at least five plants were harvested at each time point for each construct tested. After application of ethanol, a gradual decline in chl content was observed in lines expressing dsRNA constructs (Figure 5), whereas chl content in ethanol-treated control plants increased over the time course of the experiment as a result of the developmental control of chl biosynthesis. In case of *alc-dsChl I* plants, the progressive loss of chl became apparent as early as 24 hpi, while ethanol-treated *alc-dsGSA* plants lost their chl more slowly. This is in good accordance with the temporal differences in phenotype development of the two mutants. Seven days post-induction, chl content in *alc-dsChl I* plants was only approximately 3% of that before treatment, whereas chl content in phenotypically affected leaves of *alc-dsGSA* was reduced to levels of about 20% of those before treatment.



**Figure 5.** Changes in chl content in *alc-dsRNA* transgenic plants after ethanol induction.

Control plants (○), *alc-dsGSA* line 60 (●), and *alc-dsChl I* line 16 (▼) were induced with 100 ml 1% (v/v) ethanol via root drenching, and chl content was measured at the times indicated. Samples were taken from leaves being c. 5 cm in size at the time of induction until 7 dpi. The chl content is expressed as a value relative to that at day 0. Bars show the SD of five replicates.



**Figure 6.** Temporal and spatial control of *Chl I* gene silencing.

(a, b) Wild-type (a) and *alc-dsChl I* (b) plants (line 16) were root-drenched with 1% ethanol solution every 2 days. The pictures were taken at 15 dpi. (c, d) Local *Chl I* silencing. The leaf indicated by an arrow was enclosed with 3 ml of 4% (v/v) ethanol for 48 h. (c), wild-type control plant; (d), *alc-dsChl I* plant from line 16.

### Spatial control of gene silencing using the *alc* system

Previous studies demonstrated that spatial induction of *alc*-reporter gene constructs could be achieved by exposing single leaf to ethanol vapor (Sweetman *et al.*, 2002). In order to test whether this was also possible for inducible gene silencing, an individual leaf of an *alc-dsChl I* plant was exposed to ethanol vapor using a similar 'bagging' experiment as described before by Sweetman *et al.*, (2002). As shown in Figure 6(d), the respective leaf displayed the typical symptoms of *Chl I* downregulation observed in whole plant induction experiments. The phenotype was restricted to the treated leaf only, indicating that neither transport of ethanol into adjacent parts of the plant nor spread of silencing occurred. Thus, our data demonstrate that confined vapor treatment allows for spatial control of gene silencing using the *alc* system.

### Discussion

Downregulation of endogenous genes via PTGS using sense or antisense constructs is a crucial tool to assess gene function in transgenic plants. Recent findings indicate that expression of self-complementary hairpin RNAs greatly enhances the efficacy of such experiments (Chuang and Meyerowitz, 2000; Levin *et al.*, 2000; Smith *et al.*, 2000; Stoutjesdijk *et al.*, 2002; Waterhouse *et al.*, 1998). Although

these approaches have been proven to be extremely useful, they are not without problems. One drawback of these studies is that constitutive gene silencing often entails pleiotropic effects on growth and development of the transgenic plants, which complicate the interpretation of the phenotype and might mask true gene function. Furthermore, if expression of the target gene is essential for early growth or regeneration during tissue culture, vital plants might not be recovered. Here, we show that temporal and spatial control of gene silencing in transgenic plants can be achieved by ethanol-inducible expression of dsRNA constructs using the *alc* system.

We used two genes involved in chl biosynthesis, namely *Chl I* and *GSA*, to test downregulation by ethanol-inducible expression of antisense fragments and dsRNA constructs, respectively. Suppression of either of the two target genes was assumed to result in a loss of pigmentation because of reduced chl biosynthesis (Höfgen *et al.*, 1994; Papenbrock *et al.*, 2000). However, only constructs giving rise to dsRNA were effective in triggering gene silencing, as revealed by rapid development of the characteristic phenotype after induction with ethanol. Constructs designed to express conventional antisense fragments failed to interfere with expression of the target gene, which was further confirmed by Northern blotting. This is consistent with the assumption that dsRNA is a much stronger trigger of PTGS than ssRNA, possibly by circumventing the initial conversion of ssRNA into dsRNA by an RNA-dependent RNA polymerase (Béclin *et al.*, 2002; Dalmay *et al.*, 2000). First signs of phenotype development occurred at 36 hpi for *alc-dsChl I* to 48 hpi for *alc-dsGSA*, most likely reflecting differences in mRNA and protein turnover rates of the respective endogene. The time point of phenotype development was highly reproducible in several independent induction experiments (data not shown) and is much more rapid than the 10 days to 3 weeks, which have been reported to be necessary for phenotype induction using various virus-based gene silencing systems (Gosselé *et al.*, 2002; Hiriart *et al.*, 2002; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002). Using a single induction, the phenotype persisted for approximately 9 days, which is considerably shorter than what has been shown for VIGS (Gosselé *et al.*, 2002; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002). However, re-iterated ethanol treatment permits to maintain stable silencing for extended periods, which should enable to follow the full sequence of consequences of reduced gene expression whenever desirable.

One of the most interesting features of gene silencing is that it can act non-cell-autonomously, meaning that it can be induced locally and subsequently spread throughout the organism, implying the existence of a mobile silencing signal (Klahre *et al.*, 2002; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). Little is known about the nature of the signal, but it seems likely that the sequence-specific component is

an RNA (Boutla *et al.*, 2002; Mlotshwa *et al.*, 2002). However, evidence suggests that highly expressed transgenes are much better for systemic silencing than are endogenous genes, suggesting that the amount of target RNA is important in establishing systemic silencing in response to the mobile signal (Palauqui and Vaucheret, 1998; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). This is consistent with the finding that using the *alc* system, local silencing of *Chl I* could be achieved by confined ethanol treatment of a single leaf. In this case, no other parts of the plant but the treated leaf displayed any visible signs of *Chl I* silencing, indicating that no systemic spread of PTGS occurred. Spatial control of gene silencing is desirable in some situations, for instance to study the function of widely expressed genes on an organ or tissue basis without affecting the entire plant. On the other hand, the system allows for the investigation of physiological perturbations on the whole plant level, which were caused by local silencing of a particular gene.

To determine the degree of silencing, which could be achieved by inducible expression of dsRNA constructs on the biochemical level, the decline in chl content in phenotypically affected leaves was taken as an indirect measure for chl biosynthetic capacity. A reduction in chl of approximately 80% was achieved in *alc-dsGSA* plants, which is in the range of what has been reported for transgenic tobacco plants constitutively expressing *GSA* antisense RNA (Höfgen *et al.*, 1994). Transgenic tobacco plants constitutively silenced for *Chl I* were shown to have approximately 40% of the chl content as compared to the control (Papenbrock *et al.*, 2000). Inducible silencing of *Chl I* using the *alc* system reduced the chl content in phenotypically affected leaves to approximately 3% of that before induction, indicating a much stronger effect on *Chl I* expression than that in constitutively silenced plants. It is reasonable to assume that in case of constitutive silencing, there is a considerable selection against the reduction of Mg-chelatase activity below a certain threshold level, which does not support plant growth under autotrophic conditions. Thus, in some cases, inducible PTGS using the *alc* system is likely to provide a valid strategy to obtain a degree of silencing, which otherwise prevents the regeneration of viable transgenics.

Recently, an alternative system to achieve chemical-regulated inducible gene silencing has been described, which takes advantage of 17 $\beta$ -estradiol-inducible recombination to trigger the expression of an intron-containing inverted-repeat RNA (CLX system; Guo *et al.*, 2003). In this case, induction of the system leads to permanent activation of PTGS mimicking expression from a constitutive promoter. However, because of the nature of the inducer, the CLX system might not be readily applicable to soil-grown plants, and thus its use in physiological studies is limited.

In summary, the data presented here demonstrate the utility of the *alc* gene system to achieve transient gene

silencing by inducible expression of dsRNA constructs. The *alc* system offers an enormous flexibility with respect to time point of induction, expression level, spatial control, and duration of expression, and is applicable to a variety of plant species. In contrast to other chemically regulated systems, ethanol, or alternatively acetaldehyde, is comparatively a benign inducer and exerts only minimal physiological side-effects in concentrations necessary for induction (Junker *et al.*, 2003). These attributes greatly enhance the reproducibility of silencing experiments, which is of particular importance in metabolic studies requiring a large population of uniformly silenced individuals. The system provides a powerful tool to investigate molecular and physiological alterations associated with repression of a target gene at temporal and spatial resolution. Thus, dissection of primary and secondary effects of gene silencing should be greatly facilitated, allowing more precise predictions of gene function.

## Experimental procedures

### Transgenic plants, growth, and maintenance

Tobacco plants (*N. tabacum* cv. Samsun NN) were obtained from Vereinigte Saatuchten eG (Ebsdorf, Germany) and grown in tissue culture under a 16-h light/8-h dark regime (irradiance 150  $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ ) at 50% humidity on Murashige and Skoog medium (Sigma, St Louis, MO, USA) containing 2% (w/v) sucrose. Plants in the greenhouse were kept in soil under a light/dark regime of 16 h light (25°C) and 8 h (20°C) dark.

### Plasmid construction and plant transformation

All constructs for plant transformation were cloned into p35S:*alcR*, a derivative of pBin19 (Bevan, 1984), carrying the *alcR* gene from *A. nidulans* between the CaMV 35S promoter and the *nos* terminator (Caddick *et al.*, 1998) using standard procedures (Sambrook *et al.*, 1989). Fragments containing portions of the respective target gene in sense and antisense orientation separated by an intron were initially assembled into a pUC-based vector. To this end, the first intron of the gibberellin 20 (GA20) oxidase gene from *Solanum tuberosum* (kindly provided by S. Biemelt, IPK Gatersleben, Germany) was PCR amplified using the primers 5'-cctgcaggctcgagactagatctggtacggaccgtactactcta-3' and 5'-cc-tgcagggtcgactctagagatccctatataatttaagtggaaa-3'. The oligonucleotides were designed to introduce *PstI/XhoI/Spel/BglII* sites at the 5' end and *BamHI/XbaI/SaI/PstI* sites at the 3' end, into the resulting PCR product. The 200-bp intron fragment was inserted into a pUC18 vector devoid of the polylinker site via blunt-end ligation resulting in the plasmid pUC-RNAi. A 655-bp fragment of the *N. tabacum Chl I* gene (GenBank Accession number U67064) comprising nucleotides 454–1108 was amplified by PCR from tobacco cDNA and inserted as a *BamHI/SaI* fragment in sense orientation downstream of the GA20 intron into pUC-RNAi using the before-mentioned restriction sites. The same fragment was inserted in antisense orientation into the *BglII/XhoI* sites of pUC-RNAi already carrying the *Chl I* sense fragment. Subsequently, the entire fragment comprising sense and antisense fragments of *Chl I* interspersed by the potato GA20 oxidase intron was excised from

pUC-RNAi using the flanking *PstI* restriction sites and inserted into a pUC-based plasmid between a chimeric *alcA* promoter and a *nos* terminator sequence (Caddick *et al.*, 1998). The resulting *alcA* expression cassette was subsequently inserted into the *HindIII* site of p35S:*alcR* yielding the construct *alc-dsChl I*. An 804-bp fragment of *N. tabacum GSA* (Höfgen *et al.*, 1994; GenBank Accession number X65974) comprising nucleotides 298–1101 of the respective cDNA clone, was amplified by PCR and manipulated as described above to form plasmid *alc-dsGSA*.

To obtain constructs for ethanol-inducible antisense RNA expression, the same fragments as used before were linked in reverse orientation to the chimeric *alcA* promoter, and the entire cassette was subsequently ligated into p35S:*alcR* as above.

Transformation of tobacco plants by *Agrobacterium*-mediated gene transfer using *A. tumefaciens* strain C58C1:pGV2260 was carried out as described previously by Rosahl *et al.*, (1987).

Constructs and biomaterials concerning the ethanol-inducible system are available for academic research purposes subject to satisfactory completion of a material transfer agreement with Syngenta. For further information, contact the Licensing Manager, Syngenta; Jeallots Hill International Research Center, Bracknell, Berkshire RG42 6EY, UK.

### Ethanol induction

Plants (42 days old) cultivated in the greenhouse in 2.5-l pots were induced with 100 ml of 1% (v/v) ethanol solution via root drenching. Normal watering was resumed after application. Samples for RNA, chl, and protein analysis were taken at various time points indicated in the section under Results. If not otherwise stated, young leaves, being approximately 5 cm at the time point of induction, were followed over the time course of the experiment. For spatial induction, an individual leaf was enclosed in a 15 cm  $\times$  10 cm transparent plastic bag with 3 ml of 4% (v/v) ethanol as described previously by Sweetman *et al.*, (2002). The bag was removed after 48 h and phenotype development was monitored by eye.

### RNA analysis

Total RNA was extracted from tobacco leaf material as described by Logemann *et al.*, (1987), and 30  $\mu\text{g}$  per sample was separated on a 1.5% (w/v) formaldehyde-agarose gel using conditions described by Sambrook *et al.*, (1989). After electrophoresis, RNA was transferred to a nitrocellulose membrane (GeneScreen, NEN Life Science Products, Boston, USA) and fixed by UV cross-linking. Filters were pre-hybridized, hybridized, and washed essentially as described by Sweetman *et al.*, (2002). *GSA* and *Chl I* transcripts were detected using a random-primed [ $^{32}\text{P}$ ]-labeled cDNA fragment.

### Protein analysis

Protein extracts were prepared by homogenization of leaf material in a buffer containing 25 mM HEPES, pH 7.0, 12 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 8 mM DTT, 10  $\mu\text{M}$  PMSF, 0.1% Triton, and 10% glycerol. Protein content was determined according to Bradford (1976). After heat denaturation, 30  $\mu\text{g}$  of total protein was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membrane (Poreblot, Macherey und Nagel, Düren, Germany). Immunodetection was carried out using the ECL kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer, using a rabbit



anti-GSA primary antibody (kindly provided by Dr Bernhard Grimm, Humboldt University, Berlin, Germany) and peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA).

### Chlorophyll determination

Chlorophyll was measured in ethanol extracts and concentrations were determined as described by Lichtenthaler (1987).

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Marina Byzova · Christoph Verduyn · Dirk De Brouwer  
Marc De Block

## Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner

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**Abstract** Oilseed rape (*Brassica napus* L.) genotypes with no or small petals are thought to have advantages in photosynthetic activity. The flowers of field-grown oilseed rape form a bright-yellow canopy that reflects and absorbs nearly 60% of the photosynthetically active radiation (PAR), causing a severe yield penalty. Reducing the size of the petals and/or removing the reflecting colour will improve the transmission of PAR to the leaves and is expected to increase the crop productivity. In this study the 'hairpin' RNA-mediated (hpRNA) gene silencing technology was implemented in *Arabidopsis thaliana* (L.) Heynh. and *B. napus* to silence B-type MADS-box floral organ identity genes in a second-whorl-specific manner. In *Arabidopsis*, silencing of B-type MADS-box genes was obtained by expressing *B. napus* *APETALA3* (*BAP3*) or *PISTILLATA* (*BPI*) homologous self-complementary hpRNA constructs under control of the *Arabidopsis* A-type MADS-box gene *APETALA1* (*API*) promoter. In *B. napus*, silencing of the *BPI* gene family was achieved by expressing a similar hpRNA construct as used in *Arabidopsis* under the control of a chimeric promoter consisting of a modified petal-specific *Arabidopsis* *AP3* promoter fragment fused to the *API* promoter. In this way, transgenic plants were generated producing male fertile flowers in which the petals were converted into sepals (*Arabidopsis*) or into sepaloid petals (*B. napus*). These novel flower phenotypes were stable and heritable in both species.

**Keywords** Apetalous · *Arabidopsis* · *Brassica* · Double sepaloid · MADS-box · Petal

**Abbreviations** PAR: photosynthetically active radiation · *ST-LS1*: potato light-inducible tissue-specific *ST-LS1* gene · *GUS*:  $\beta$ -glucuronidase

### Introduction

Flowers of oilseed rape (*Brassica napus*) have four well-developed bright-yellow petals. During flowering time, flowers form a very bright-yellow layer that reflects and absorbs solar radiation. As consequence, only 24% of the photosynthetically active radiation (PAR) reaches the leaf canopy (Chapman et al. 1984). This accelerates leaf and bract senescence, reduces dry matter accumulation, and lowers seed set (Daniels et al. 1986).

A few strategies to improve the photosynthetic efficiency of oilseed rape by utilising different apetalous variants (Buzza 1983; Jiang and Becker 2003) or the *stamenoid petal* (stap) variant with flowers bearing staminoid petals (Fray et al. 1997) have been proposed. Physiological analyses have revealed the potential benefit of such a petalless flower phenotype on *B. napus* yield (Rao et al. 1991; Fray et al. 1995).

The currently used apetalous genotypes are controlled either by two recessive genes (Fray et al. 1996) or by an interaction of cytoplasmic genes and two pairs of nuclear genes (Jiang and Becker 2003). This genetic complexity makes it difficult to fully implement the apetalous trait into commercial rapeseed varieties. Additionally, the apetalous character appears to be unstable under field conditions at high temperatures and in long days (Rao et al. 1991). The *B. napus* *stap* variant also possesses poor agronomic attributes, such as deformed leaves and poor vigour (Fray et al. 1997).

A more promising strategy to improve PAR transmission in oilseed rape would be the use of a single dominant gene that converts the bright-yellow petals into small non-light reflecting structures such as sepals. Such an organ conversion is preferable over the removal of the petals to avoid interfering with insect pollination. Pierre et al. (1996) have shown that honeybees, the main

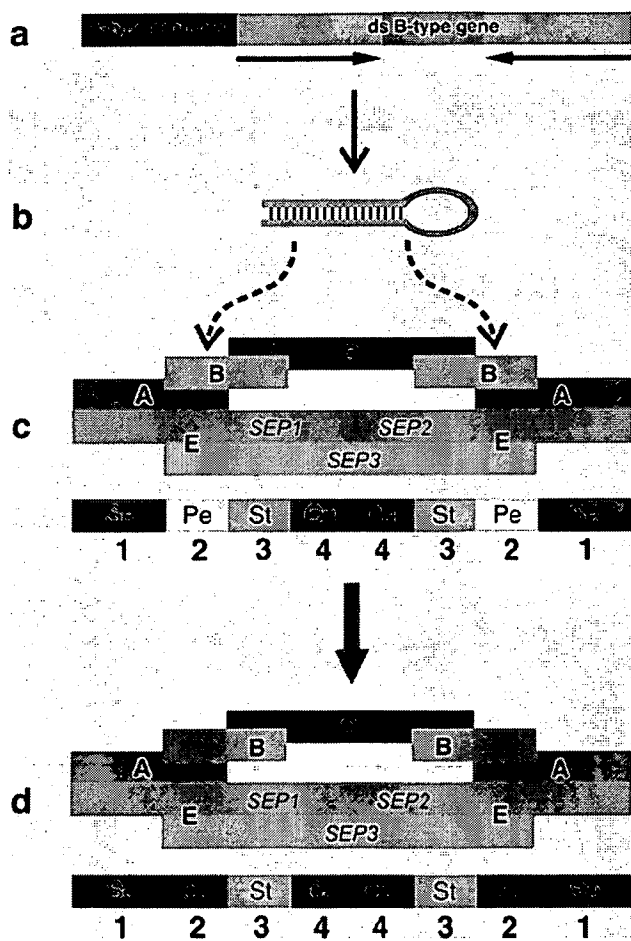
M. Byzova · C. Verduyn · D. De Brouwer  
M. De Block (✉)  
Bayer BioScience N.V., Jozef Plateastraat 22,  
9000 Gent, Belgium  
E-mail: marc.deblock@bayercropscience.com  
Fax: +32-9-2240694

pollinators on oilseed rape, do not crawl over the anthers and stigma of apetalous flowers as they do in petalous ones but often insert their tongues between the sepals to collect the nectar. In this way pollination might be reduced, resulting in a lower seed set.

The molecular mechanisms governing floral organ identity are well understood. According to the "A-E" model, the organ identity of each floral whorl is determined by a unique combination of four organ identity activities, called A, B, C and E (Weigel and Meyerowitz 1994; Pelaz et al. 2000; Jack 2001; Theissen 2001; Fig. 1). Expression of the (A)-type genes specifies sepal formation. The combination of (A + B + E) activities spec-

ifies the formation of petals, while combined (B + C + E) functions specify stamen formation. Expression of the (C + E)-type genes determines the development of carpels (Fig. 1). All types of organ identity genes have been cloned from *Arabidopsis*. An example of the A-type gene is *API* (Mandel et al. 1992). The B-type genes are *AP3* (Jack et al. 1992) and *PI* (Goto and Meyerowitz 1994), and the C-type gene is *AGAMOUS* (*AG*) (Yanofsky et al. 1990). The E-function is provided by three *SEPALLATA* genes (Pelaz et al. 2000). All these genes are transcription factors belonging to the MADS-box gene family.

In this paper, silencing of the B-type MADS-box genes in a second-whorl-specific manner was obtained in both *Arabidopsis* and *B. napus* flowers by expressing a *B. napus* B-type gene hpRNA construct under control of an *Arabidopsis* A-type MADS-box gene promoter (Fig. 1). In this way, *Arabidopsis* lines with double sepaloid flowers and *B. napus* lines with flowers in which petals are converted into sepaloid petals were generated. The novel flower phenotypes were stable and heritable in both species.



**Fig. 1a–d** Experimental strategy. **a** Representation of the basic construct used in this study. A DNA fragment of a 3'-coding region of a B-type MADS-box gene (yellow box) was sub-cloned as an inverted repeat (directions are indicated by arrows) with a part of the GUS gene or the intron IV2 from gene *ST-LS1* (Vancanneyt et al. 1990) as a spacer (blue box). The constructs were driven by an A-type MADS-box gene promoter (green box). **b** Transcripts produced by the construct are predicted to form a hairpin structure. **c** Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in wild-type plants. **d** Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in transgenic plants. In transgenic plants, down-regulation of the B-type function in the second whorl only leads to development of sepals instead of petals. Numbers indicate whorls. *Se* sepals, *Pe* petals, *St* stamens, *Ca* carpels

## Materials and methods

### Plant material

*Arabidopsis thaliana* (L.) Heynh. ecotype C24, kindly provided by Dr. M. Van Lijsebettens (VIB, Gent, Belgium), and the double haploid *Brassica napus* L. line cv. Simon (Bayer BioScience N.V., Gent, Belgium) were used in this study.

### Plasmid construction

The 3'-coding regions of the *BAP3* and *BPI* genes were cloned by means of RT-PCR performed on total RNA isolated from *B. napus* flower buds. RT-PCR was performed according to the protocol of the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). *AP3* cDNA-specific primers:

- 5'-CGCACTCAGATTAAGCAGAGGC-3' and
- 5'-GGAAGGTAATGATGTCAGAGGC-3'

and *PI* cDNA-specific primers:

- 5'-GGGAGAAGATATACAGTCTCTCAAC-3' and
- 5'-GAATCGGTTGCACTCTATATCC-3'

were chosen based on the published sequences (Jack et al. 1992, GenBank Accession D30807; Goto and Meyerowitz 1994, GenBank Accession M86337). In the pAPI::hpBAP3 construct, one of the *BAP3*-specific DNA fragments, 380-bp in length, was cloned as an inverted repeat with the  $\beta$ -glucuronidase (GUS) fragment containing nucleotides 744–975 as a spacer. In the pAPI::hpBPI construct, one of the *BPI*-specific fragments, 255-bp in length, was cloned as an inverted repeat with the intron IV2 from the potato light-inducible tissue-specific gene *ST-LS1*, 251-bp in length, as a spacer (Vancanneyt et al. 1990). In the pAPI::hpBAP3 and pAPI::hpBPI constructs, gene-specific structures were driven by a 1,182-bp fragment of the *API* promoter. The fragment of the *API* promoter (–1182 to +1) was cloned by means of PCR from pKY65 plasmid kindly provided by Martin Yanofsky. In p $\Delta$ AP3-*API*::hpBPI fragments of the *AP3* promoter, containing nucleotides –727 to –556 and –224 to –1 were cloned by PCR based on

the published sequence (Irish and Yamamoto 1995, GenBank Accession U30729) and linked to the 5'-end of the *API* promoter. Plasmid constructs were introduced into *Agrobacterium tumefaciens* strain C58C1rif by electroporation.

#### Plant transformation

The transformations of *A. thaliana* and *B. napus* were essentially done as described by Valvekens et al. (1992) and De Block et al. (1989), respectively.

#### Cytology

The embedding was done in Histo-resin as advised by the manufacturer (Leica, Heidelberg, Germany). Sections 5 µm thick were stained with 0.05% toluidine blue.

#### In situ hybridization

Embedding in methacrylate, sectioning, and the removal of the plastic were essentially done as described by Baskin et al. (1992). The in situ hybridizations on 7-µm sections were essentially done as described by De Block and De Brouwer (1993).

#### Microscopy

Sections were examined with an Axioplan (Zeiss, Jena, Germany) microscope equipped with Normaski differential interference contrast.

#### Spectrophotometric determination of chlorophyll

The total chlorophyll (*a* + *b*) content was measured as described by Bruisma (1963).

## Results

### General strategy: silencing the B-type MADS-box genes in a second floral whorl-specific manner

To convert petals into sepals without interfering with anther development, the strategy outlined in Fig. 1 was used. Following the A–E flower development model it is expected that silencing of a B-type MADS-box gene, *AP3* or *PI*, in the second whorl will redirect the development of petals into sepals. This could be obtained by expressing in the second, but not in the third whorl self-complementary 'hairpin' RNA (hpRNA) constructs containing *AP3*- and/or *PI*-specific sequences. Down-regulation of the B-type MADS-box genes in the third whorl has to be avoided to maintain normal male fertility. For this purpose an A-type promoter driving the expression of the hpRNA construct could be used.

Starting from the *PI* and *AP3* sequences (Jack et al. 1992; Goto and Meyerowitz 1994), we identified in the amphidiploid *B. napus* five *AP3*-like (*BAP3*) and three *PI*-like (*BPI*) genes that were actively expressed during flower development (data not shown). Fragments of the 3'-coding region of the *BAP3* and *BPI* genes were isolated. The nucleotide sequence similarity between

members of the same B-type MADS-box gene subfamily turned out to be on average 95%. Each *B. napus* gene subfamily shared with its unique *Arabidopsis* counterpart about 91% sequence similarity, containing multiple blocks of more than 20 bases of perfect homology. This high sequence similarity should be sufficient to silence the target genes in both *Arabidopsis* and *B. napus* by using the same hpRNA constructs (Helliwell and Waterhouse 2003). The feasibility of the strategy to convert petals into sepals by silencing the B-type MADS-box genes only in the second floral whorl was first evaluated in the model plant *Arabidopsis thaliana*.

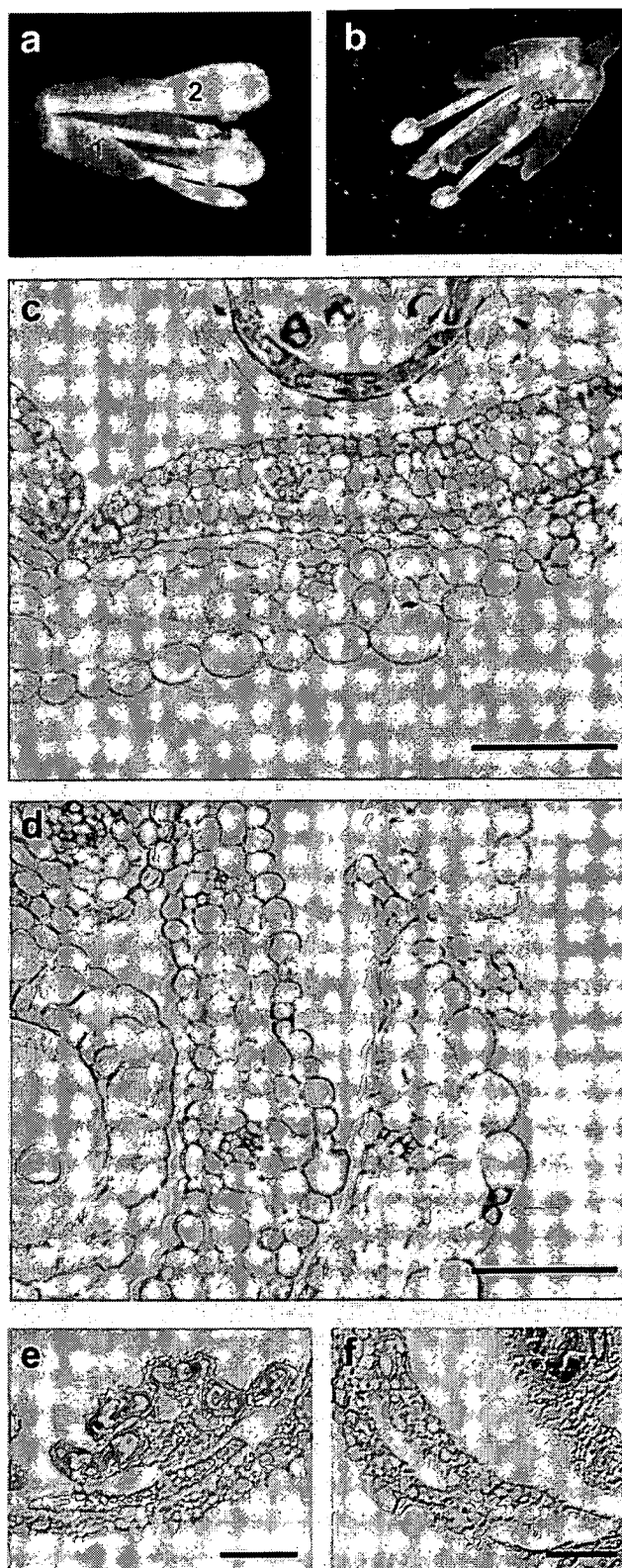
### Generation of *Arabidopsis* transgenic lines with male fertile double sepaloid flowers

To make constructs that produce hpRNA B-type MADS-box gene transcript, the 3'-coding regions of one *BAP3* and one *BPI* gene, were subcloned as an inverted repeat (see Materials and methods). Both hpBAP3 and hpBPI gene-specific sequences were driven by a 1.1-kb promoter fragment of the *Arabidopsis API* gene. The resulting pAPI::hpBAP3 and pAPI::hpBPI constructs were introduced separately into *Arabidopsis*.

A total of 125 pAPI::hpBAP3 and 56 pAPI::hpBPI transgenic lines was generated. All the plants were normal in terms of vegetative growth while they had morphological changes in flower organs. 16.9% of the pAPI::hpBPI and 5.6% of the pAPI::hpBAP3 lines exhibited the desirable double sepaloid phenotype (Fig. 2b). Instead of petals, sepals developed in the second floral whorl, indistinguishable from those of the first whorl except for their slightly smaller size. Despite their transformation, these organs developed in the positions and on a time course characteristics of petals. Some other pAPI::hpBAP3 *T*<sub>0</sub> plants had a range of phenotypes related to the severity of homeotic transformations observed in petal and stamen development. 10.4% of the pAPI::hpBAP3 lines produced flowers with short white petals and 20% of the lines had homeotic aberrations in stamens ranging from weak carpelloidy to complete transformation of stamens into carpels (Table 1). In contrast to the pAPI::hpBAP3 lines, no aberrations in the third floral whorl were observed in the pAPI::hpBPI transgenic plants (Table 1).

Microscopic analysis of cross-sections of mature pAPI::hpBPI double sepaloid flowers revealed that the mesophyll cells of the second-whorl organs were sepaloid in nature, as indicated by the presence of chloroplasts and their larger size than those normally found in wild-type petals. The abaxial epidermis was like that of sepals, consisting of stomata and irregularly shaped cells (Fig. 2d). The same results were obtained for pAPI::hpBAP3 double sepaloid flowers (data not shown).

To confirm that the double sepaloid phenotype of *Arabidopsis* transgenic plants was caused by depletion of expression of endogenous B-type homeotic genes in the



second whorl, the *PI* mRNA expression pattern in pAP1::hpBPI was examined by in situ hybridization. In wild-type *Arabidopsis* flowers, *PI* mRNA is detected

**Fig. 2a–f** Analysis of the double sepaloid pAP1::hpBPI *Arabidopsis thaliana* flowers. **a** C24 *Arabidopsis* wild-type mature flower. **b** Mature transgenic flower. Second-whorl organs are sepals (arrow) that are slightly smaller than the true sepals. **c, d** Cytological transverse sections taken approximately in the middle of anthers of flower buds at stage 12. **c** Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of the second-whorl petals are smaller than those of sepals developed in the first whorl. Abaxial epidermal cells of petals are regular in shape. **d** Cellular morphology of first- and second-whorl organs of a transgenic flower. Mesophyll and epidermal cells of the second-whorl organs are slightly smaller in size than cells of the first-whorl sepals. The shape of the cells of the second-whorl sepals is similar to those of the first-whorl sepals. Stomata (arrows) are present in the abaxial epidermis of the second-whorl organs as in normal first-whorl sepals. **e, f** In situ analysis of *PI* expression in transverse sections of wild-type and transgenic flowers. The hybridisation signal is confined to the second- and the third-whorl organs in wild-type flowers (**e**). In transgenic flowers (**f**) *PI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 50  $\mu$ m (**c, d, f**), 100  $\mu$ m (**e**)

from stage 3 (Smyth et al. 1990) in second- third- and fourth-whorl primordia. In the second and third whorls it persists until anthesis (Goto and Meyerowitz 1994). In pAP1::hpBPI double sepaloid flowers the *PI* transcript was not detectable in the second-whorl primordia at any of the stages examined (Fig. 2f). Expression of *PI* in developing stamens was similar to that observed in the wild type.

As it has been shown that expression of both *PI* and *AP3* genes is reduced when either the *PI* or *AP3* gene is mutated (Jack et al. 1992; Goto and Meyerowitz 1994), we anticipated that inhibition of expression of one of the B-type MADS-box genes in a tissue where both genes are active would lead to reduction of expression of the counterpart gene in the same manner. To verify this hypothesis, in situ hybridization of the pAP1::hpBPI flowers using the *BAP3*-specific fragment as a probe was performed. As was predicted, *AP3* RNA was not detected in the second whorl of developing organs. However, no reduction in the level of the *AP3* mRNA was observed in stamens (data not shown).

Heritability and stability of the double sepaloid trait was tested by self-pollination. The trait was heritable and in the case of pAP1::hpBPI stable through the T<sub>1</sub> and T<sub>2</sub> generations. In the case of pAP1::hpBAP3 some T<sub>1</sub> and T<sub>2</sub> lines produced flowers with homeotic aberrations in stamens, as previously observed in the T<sub>0</sub> plants.

In *B. napus*, silencing of B-type MADS-box genes in the second whorl results in the transformation of petals into sepaloid petals

To evaluate whether the expression of the pAP1::hpBAP3 and pAP1::hpBPI genes would also result in a double sepaloid phenotype in *B. napus*, 48 and 53 transgenic lines, respectively, were generated.

All the pAP1::hpBAP3 lines had wild-type flowers. Among the pAP1::hpBPI transgenic lines, 22.6%

**Table 1** Phenotypic analysis of T<sub>0</sub> *Arabidopsis thaliana* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)		
		Double sepaloid Fertile	Double sepaloid Partially male sterile <sup>a</sup>	Short petals Fertile
pAPI::hpBPI	56	16.9	< 2	18.9
pAPI::hpBAP3	125	5.6	20	10.4

<sup>a</sup>Range of aberrations in stamens from mild to complete conversion of stamens into carpels

exhibited an apetalous or partially apetalous phenotype characterised by the appearance of flowers without petals or bearing 1, 2 or 3 petals only (Table 2). Frequently, the petals were significantly smaller and narrower than those from wild type (data not shown). However, this phenotype was unstable and not heritable.

The absence of the double sepaloid phenotype in transgenic *B. napus* lines with the same constructs used in *Arabidopsis* could be due to an inability of the *Arabidopsis* *API* promoter to direct transcription of adequate amounts of double-stranded transcripts necessary to trigger silencing of all target *BAP3* or *BPI* genes expressed in rapeseed flowers. Starting from this hypothesis, a new construct was generated that could produce higher amounts of hpRNA. Because the pAPI::hpBAP3 *B. napus* transgenic plants did not exhibit any phenotypes different from those of wild-type plants, and in *Arabidopsis* the most stable double sepaloid flower phenotype was obtained with the hpBPI construct, we continued only with the hpRNA *BPI* gene.

To enhance the level of expression of hpBPI specifically in the second whorl, an *Arabidopsis* modified *AP3* regulatory fragment was added to the *API* promoter.

Discrete *cis*-acting elements regulating spatial and temporal expression of the *Arabidopsis* *AP3* gene have been identified (Hill et al. 1998; Tilly et al. 1998). Based on these data the positive regulator of the *AP3* expression during the early stages of flower development was combined with the petal-specific regulatory region (see Materials and methods). The modified *AP3* promoter was introduced in the pAPI::hpBPI construct directly upstream of the *API* sequence. This pΔAP3-API::hpBPI construct was transformed into *B. napus*.

Of the 125 primary transformants, 11.2% produced flowers with aberrant second-whorl organs. Of these 11.2% lines, half (5.6%) produced flowers in which petals were converted into sepaloid petals (Fig. 3a, Table 2). These organs were yellowish-green, indicating the presence of chloroplasts in their cells that is characteristic of wild-type sepals. The size of the sepaloid petals was comparable to the size of true sepals. These organs

were narrow and almost strap-like in shape, like sepals, but had a small lamina and base, characteristic of a petal. In addition the lamina portion was wrinkled (Fig. 3a).

The aberrant *B. napus* flowers with sepaloid petals were analysed microscopically to verify the identity of tissues in the second-whorl organs. As shown in Fig. 3c the size and the shape of epidermal and mesophyll cells of these organs were indistinguishable from the first-whorl sepals. Moreover, the mesophyll cells of the sepaloid petals contained a large number of chloroplasts (Fig. 3d).

In addition, spectrophotometric analysis of chlorophyll fluorescence, which was done on the first and the second floral organs of transgenic plants, revealed that chlorophyll content in the sepaloid petals is only 30% less than in the true wild-type sepals (data not shown).

In situ hybridization of flower sections with a *BPI*-specific probe confirmed the absence of a detectable level of *BPI* gene expression in the second whorl of the transgenic flowers, indicating that the complete *BPI* gene family was down-regulated (Fig. 3e).

The other half of the 11.2% transgenic pΔAP3-API::hpBPI lines exhibited partial apetalous and apetalous phenotypes similar to those observed in pAPI::hpBPI transgenic plants (Table 2).

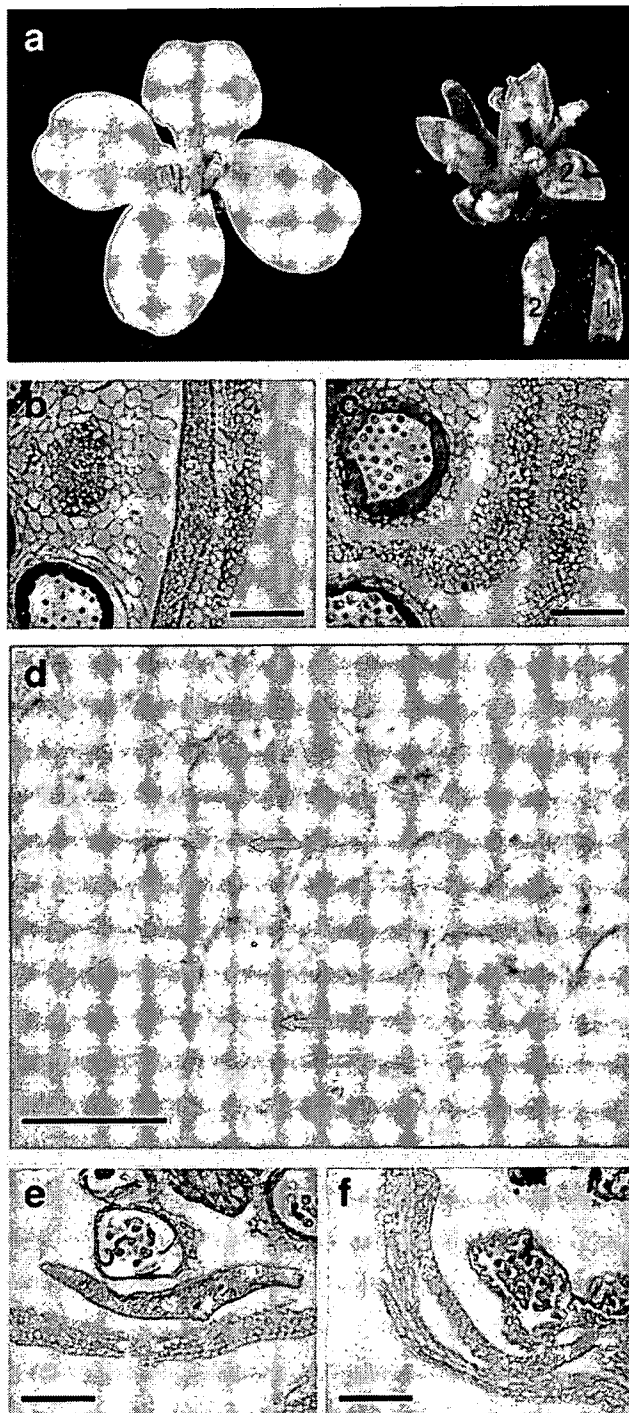
The flower phenotype with sepaloid petals is a stable trait in *B. napus* transgenic plants

The stability of transformation of petals to sepaloid petals in *B. napus* was tested for six lines, of which the original T<sub>0</sub> plants had flowers with sepaloid petals and contained only one copy the pΔAP3-API::hpBPI transgene. The T<sub>0</sub> plants were first maintained by selfing. The transgenic plants of these T<sub>1</sub> generations had flowers with sepaloid petals while the azygous segregants had normal wild-type flowers. For each line ten transgenic plants of the T<sub>1</sub> generation were backcrossed with the original non-transgenic double haploid *B. napus* line cv.

**Table 2** Phenotypic analysis of T<sub>0</sub> *Brassica napus* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)	
		Sepaloid petals	Apetalous/partially apetalous
pAPI::hpBAP3	48	0	0
pAPI::hpBPI	53	0	22.6
pΔAP3-API::hpBPI	125	5.6	5.6





Simon. Depending on whether the  $T_1$  plant used was homo- or heterozygous for the transgene, all or 50% of the  $F_1$  plants, respectively, had sepaloid petals in their flowers. A second backcross was done with 15 plants of each line. As expected, in the  $F_2$  generations there was a 1:1 segregation of wild-type plants and plants with sepaloid petals. The flower phenotype of the transgenic  $F_2$  plants was identical to those of the  $T_0$ ,  $T_1$  and  $F_1$  transgenic plants.

**Fig. 3a–e** Analysis of the pΔAP3-AP1::hpBPI *B. napus* flowers. **a** Morphological features of *Brassica napus* flowers: mature wild-type flower (left), mature flower of a transgenic plant (right). The second-whorl organs of a transgenic flower are yellowish-green sepaloid petals (arrow). The size of these organs is similar to sepals developed in the first whorl, but the lamina-base structure can still be distinguished (for comparison see the detached organs in the bottom right corner: the second-whorl organ (left), the first-whorl organ (right) of a transgenic flower). **b–d** Cytological transverse sections taken approximately in the middle of anthers at the early yellow bud stage (Smith and Scarisbrick 1990). **b** Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of petals are smaller than those of sepals. Epidermal cells of petals are regular in shape. **c** Cellular morphology of the first- and second-whorl organs of a transgenic plant. The shape and the size of mesophyll and epidermal cells of the second-whorl organs are similar to those of the first-whorl sepals. **d** Cytology of a sepaloid petal showing the presence of chloroplasts (two examples indicated by arrows) in the mesophyll cells. **e, f** In situ analysis of *BPI* expression on transverse sections of wild-type and transgenic flowers. The hybridization signal is confined to the second- and the third-whorl organs in wild-type flowers (**e**). In transgenic flowers (**f**) *BPI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 20 μm (**d**), 100 μm (**b, c**), 200 μm (**e, f**)

## Discussion

The hpRNA-mediated gene silencing technology has been proven to be a very efficient tool for gene discovery and functional genomics in diverse organisms such as fungi (Pickford et al. 2002), nematodes (Bargmann 2001), and animals (Harborth et al. 2001). In plants this technology has been used successfully to generate virus resistance (Waterhouse et al. 1998) as well as to obtain consistent and profound inhibition of the expression of transgenes and endogenous genes (Levin et al. 2000; Smith et al. 2000; Wesley et al. 2001; Liu et al. 2002).

Chuang and Meyerowitz (2000) demonstrated that the hpRNA-mediated silencing technology could be used to interfere with flower development. A range of aberrant flower phenotypes was obtained by down-regulating the floral organ genes *AGAMOUS*, *CLAVATA3*, *APET-ALAI*, and *PERIANTHIA* using hpRNA constructs driven by the constitutive 35S and nopaline synthase promoters. Recently, it has been shown that the hpRNA-mediated silencing technique can be used to silence genes in an organ-specific way. The fatty acid composition of *Arabidopsis* and cotton seeds was modified by down-regulating the seed expression of two fatty acid desaturase genes using hpRNA constructs driven by seed-specific promoters (Liu et al. 2002; Stoutjesdijk et al. 2002).

In this article we present for the first time to our knowledge implementation of the hpRNA-mediated technology to silence a multigene family in a floral whorl-specific manner. Silencing of the B-type MADS-box genes that are present in single copy in *Arabidopsis* but are present in multiple copies in *B. napus* causes complete transformation of petals to sepals in *Arabidopsis* and partial transformation in *B. napus*. This flower phenotype is stable and heritable in both species.



In *Arabidopsis*, unlike the silencing of the *PI* gene, silencing of the *AP3* gene results in homeotic aberrations in anthers in 20% of the cases. This implies that in these lines partial silencing of the *AP3* gene also occurs in developing stamens. These results can be attributed to two possibilities. First, in wild-type *Arabidopsis* flowers *API* is expressed during early floral stages throughout all four whorls and is down-regulated in whorls 3 and 4 by the *AG* gene during stage 3, persisting in whorls 1 and 2 only (Mandel et al. 1992; Bowman et al. 1993). However, in contrast to the endogenous promoter, the smaller *API* promoter fragment we used might have some activity in the central whorls after stage 3 as proposed by Yun et al. (2002). The activity of the pAPI::hpBAP3 gene might have led to down-regulation of *AP3* in the third floral whorl. Alternatively, an aberrant stamen development in the pAPI::hpBAP3 transgenic plants might be the result of the spreading of a silencing signal between floral whorls.

Both hypotheses imply that a certain amount of dsRNA of the *AP3* gene present in the third whorl of transgenic flowers is sufficient to trigger silencing of *AP3*. This is not the case for the *PI* gene, for which the down-regulation did not result in aberrant anther phenotype. *PI* and *AP3* are both expressed in developing petals and stamens. However *PI* expression levels are similar in both whorls, whereas *AP3* expression is lower in developing stamens than in petals (Zhou et al. 2002). It may be that for this reason a lower threshold concentration of hpRNA is required in stamens to provoke a partial inhibition of the *AP3* gene expression.

Although systemic spreading of silencing may be a concern for implementation of the hpRNA-mediated silencing technology in tissue-specific applications in plants (Wang and Waterhouse 2002), the stability of the aberrant flower phenotype throughout development of our transgenic plants indicates that at least in the case of the B-type MADS-box genes there is no significant spreading of silencing between the meristems of adjacent floral organs.

Another phenomenon that might limit application of the hpRNA gene silencing technique is spreading of RNA targeting. During this process spreading of the RNA silencing signal occurs from the initial target sequence into the adjacent 5' and 3' regions (Jones et al. 1999; Vaistij et al. 2002). This may result in the participation of the entire transcribed region of the target gene in the RNA silencing process. As a consequence, expression of other homologous genes can be inhibited. Based on this hypothesis and the fact that different types of MADS-box genes share a high percentage of homology at the MADS-box regions (Purugganan et al. 1995), target-site spreading along the *AP3* or *PI* transcribed sequences would lead to silencing of not only *AP3* and *PI* but also of other MADS-box genes that are expressed in the developing second-whorl organs. In this case petals will be converted not only into sepals but also into organs with staminoid and/or carpeloid and/or other aberrant

structures. The absence of such phenotypes in our transgenic plants suggests that silencing of B-type MADS-box genes was not associated with the spreading of RNA targeting. The absence of the target-site spreading process was also observed by Vaistij et al. (2002) for the ribulose-1,5-bisphosphate carboxylase/oxygenase and phytoene desaturase genes. These results demonstrate that the hpRNA-mediated gene silencing technology can be applied not only to silence all genes of a multigene family but also to silence specifically a single member of a subfamily or even of a multigene family.

*B. napus* plants transformed with the improved pΔAP3-API::hpBPI construct have small yellowish-green sepaloid petals in the second whorl. Although mesophyll and epidermal cells of these sepaloid petals are sepaloid in morphology, the light-yellow colour suggests that some petal-specific biochemical pathways are still active in the cells of these organs. In addition, the small lamina and base of these organs are petal characteristics. It might be that undetectable levels of *BPI* transcripts are still sufficient for maintenance of some petaloid features.

Recently, in *Arabidopsis* an alternative approach was used to interfere with the expression of *AP3* in a second-whorl-specific manner (Guan et al. 2002). A zinc finger protein designed to bind to a region upstream of *AP3* was fused to the human transcriptional repression domain of mSIN3. When the *API* promoter was used to drive the expression of this artificial zinc finger transcription factor, flowers were obtained that were partially apetalous or that contained some sepaloid petals. Although the use of synthetic transcription factors is a promising approach to interfere with gene regulation, high expression levels of these transcription factors are probably needed to obtain a full phenotype by gene repression. Due to technical limitations the use of such artificial transcription factors is less feasible when multiple genes with redundant function, like the B-type MADS-box genes in *B. napus*, have to be repressed.

Theoretically, in *Arabidopsis* a double sepaloid flower phenotype may also be obtained by silencing the *SEP-ALLATA* genes in the second whorl (Fig. 1). However, due to the redundant function of the *SEPALLATA* genes, all three genes would have to be silenced together (Pelaz et al. 2000).

In conclusion, *Arabidopsis* and *B. napus* lines with a flower phenotype that is, respectively, double sepaloid or has sepaloid petals, and that is male fertile and stable in subsequent generations can be obtained by a hpRNA-mediated gene silencing of the *PISTILLATA* gene exclusively in the second floral whorl. Further physiological studies of *B. napus* transgenic lines will allow quantification of the effect of the flower architecture with sepaloid petals on the distribution of PAR and on other important agronomic features such as pollination and overall seed yield.

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Making a better RNAi vector for *Drosophila*: use of intron spacers

Young Sik Lee and Richard W. Carthew\*

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2153 North Campus Drive, Evanston, Illinois 60208, USA

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## Abstract

Double-stranded RNA induces sequence-specific inhibition of gene expression at a posttranscriptional level in eukaryotes (RNAi). This natural phenomenon has been developed into a tool for studying gene function in several model organisms, including *Drosophila melanogaster*. Transgenes bearing inverted repeats are able to exert an RNAi effect in *Drosophila*, but cloning difficulties and inconsistent silencing complicate the method. We have constructed a transgene containing inverted repeats separated by a functional intron such that mRNA produced by the transgene is predicted to form loopless hairpin RNA following splicing. A single copy of the transgene effectively and uniformly silences expression of a target gene (*white*) in transgenic flies. We have developed a vector that is designed to produce intron-spliced hairpin RNA corresponding to any *Drosophila* gene. The vector is under control of the upstream activating sequence (UAS) of the yeast transcriptional activator GAL4. The UAS/GAL4 system allows hairpin RNA to conditionally silence gene expression in *Drosophila* in a tissue-specific manner. Moreover, the presence of the intron spacer greatly enhances the stability of inverted-repeat sequences in bacteria, facilitating the cloning procedure.

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## 1. Introduction

The sequencing of the *Drosophila melanogaster* genome provides an exceptional opportunity to analyze the different functions governed by its genes [1]. Traditionally, genes are characterized by loss-of-function phenotypes caused by mutations that are induced randomly by chemical, physical, or insertional mutagenesis. The annotated sequence of the *Drosophila* genome enables reverse-genetic approaches to be used on a genome-wide scale to generate loss-of-function phenotypes. Targeted gene knockouts have recently been described in *Drosophila* [2,3], but this approach is laborious and does not allow for conditional silencing of gene expression.

Recently, RNA interference (RNAi) has been demonstrated to be an effective reverse-genetic approach to generating loss-of-function phenotypes. The presence of double-stranded RNA (dsRNA) causes the sequence-specific posttranscriptional silencing of a corresponding gene in a variety of organisms [4]. Thus, RNAi is used to

inactivate genes of interest and provides a powerful tool to study gene function. Injection of dsRNA into *Drosophila* embryos silences gene activity effectively, but its effect is transient and is not inherited in the next generation [5,6]. To overcome this problem, methods have been developed to express dsRNA stably in transgenic *Drosophila*. Most of these methods employ transgenes having an inverted-repeat (IR) configuration, which are able to produce dsRNA as extended hairpin RNA [7–10]. An alternative method has used a transgene that is symmetrically transcribed from opposing promoters [11]. A general problem with these methods is that transgenic lines often induce a variable RNAi silencing effect that exhibits incomplete penetrance and expressivity. Consequently, the copy number of silencing transgenes usually needs to be increased to observe uniform and complete gene silencing. Moreover, it is often difficult to make stable recombinant plasmids containing IRs in *Escherichia coli*. Introduction of a spacer sequence between the repeats helps stabilize some recombinant plasmids; but there are still significant reported stability problems.

In this paper, we describe an IR-based transgene designed such that the repeats are separated by a functional intron and thus are defined exons. We report that,

\* Corresponding author. Fax: 1-847-467-1380.

E-mail address: [r-carthew@northwestern.edu](mailto:r-carthew@northwestern.edu) (R.W. Carthew).

in *Drosophila*, the transgene is a powerful repressor of gene activity in vivo, with one copy of the transgene exerting uniformly strong silencing. We further describe a modular system combining GAL4-regulated gene expression with conditional production of the silencing dsRNA to allow systematic RNAi in *Drosophila* using IR exons. With this system, inhibition of gene activity is flexibly induced in any spatial or temporal pattern, allowing for precise disruption of gene function. This technique could potentially be a powerful and economical approach to studying gene function in *Drosophila* and to manipulating gene function in specific tissues of postembryonic individuals.

## 2. First-generation transgenic RNAi in *Drosophila*

We initially developed a method to express dsRNA as extended hairpin-loop RNA [10]. Its design was modeled on the successful application of hairpin RNAs in generating RNAi in plants and the nematode *Caenorhabditis elegans* by expression of transgenes with IR sequences. To facilitate cloning of IRs into recombinant plasmids, we placed a 5-base nonpalindromic sequence centered at the axis of dyad symmetry that was a *Sfi*I site, GGCCATCTAGGCC (Fig. 1). This allowed us to easily ligate gene fragments together in inverted orientation, and it increased the stability of the IR DNA during plasmid replication. Sequence repeats are often deleted in *E. coli* because cruciform intermediates form during replication of plasmid DNA and are excised by the *sbcBC* gene products. Insertion of nonrepetitive se-

quence greater than 4 bp in length between IRs inhibits cruciform excision during replication [12]. Recombinant plasmids were replicated in a recombination-deficient strain. No strain is guaranteed to propagate all recombinant clones, but the SURE strain (Stratagene) is deficient in *recBC sbcBC* and eliminates all known restriction systems. Other strains we used were JM103 and JM105, which are also mutant for *sbcBC*.

To construct an IR transgene, the IR fragment is first cloned into a generic high-copy plasmid vector such as pBluescript (Stratagene) by directional *Eco*RI–*Xho*I two-way ligation. Stable recombinants are selected, and then the IR fragment is shuttled from pBluescript into the *Drosophila* transformation plasmid vector pUAST [13]. We found it more difficult to directly clone IR fragments made in vitro into pUAST, which we circumvented by shuttling the fragment first through pBluescript. On the 5' side of the multicloning site, pUAST contains a *Drosophila* promoter linked to GAL4-responsive upstream activating sequence (UAS) enhancer repeats and on the 3' side of the multicloning site, pUAST contains a polyadenylation signal sequence. Recombinant plasmids are then injected with helper plasmid into *Drosophila* embryos and transformant flies are generated by standard P element transformation [14]. Cloning the IR into a UAS vector allowed us to use the modular design of the GAL4/UAS system in *Drosophila* for misexpressing transgenes. Many useful lines of *Drosophila* express the yeast GAL4 protein in a variety of cells/tissues at various stages of the fly life cycle [13]. GAL4 acts as a sequence-specific transcription activator in *Drosophila*. The GAL4 line is

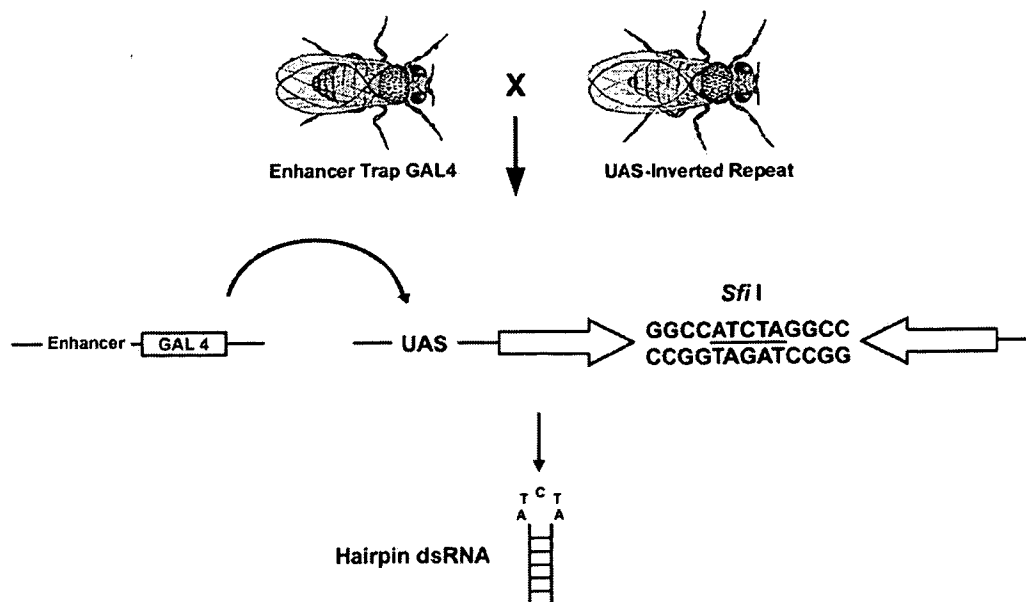


Fig. 1. First-generation transgenic RNAi. Inverted repeats are made by head–head or tail–tail ligation through a *Sfi*I site, which is introduced at one end of each repeat. The inverted repeat is placed downstream of the upstream activating sequence (UAS) promoter, and transgenic lines are made. When these UAS lines are crossed to GAL4 driver lines, the F1 heterozygotes contain both GAL4 and UAS genes [13]. Tissue-specific expression of the inverted repeats by GAL4 protein produces hairpin-loop RNA that is competent to induce RNAi in *Drosophila*.

crossed to a target UAS line carrying a single target P element inserted at a unique and random position in the genome. The target element carries a GAL4-responsive UAS enhancer, and progeny that contain both GAL4 and UAS elements express the IR sequence in cells expressing GAL4. Phenotypes due to the presence of hairpin-loop RNA in these cells can then be scored directly in flies. The RNAi system takes advantage of two very useful techniques in *Drosophila*: P element transformation and the modular GAL4/UAS system. The modular design makes analysis by RNAi flexible since hairpin-loop RNA can be produced in any spatial or temporal pattern. Moreover, RNAi is conditional, dependent on the presence of both UAS and GAL4 elements in the same individual. Thus, RNAi that might induce lethal or sterile phenotypes is conditionally generated in selected flies, and stably inherited *Drosophila* lines carrying the UAS element alone can be propagated without deleterious RNAi effect.

One feature of the target element that was critical for the success of this method was that the IR was stable in the *Drosophila* genome. This appears to be the case since UAS lines have been maintained in our lab stocks for 2 years without loss of RNAi potency when crossed with GAL4 driver lines. However, two other features of the method have proved to be more problematic. First, RNAi silencing is frequently variable, with only a fraction of treated animals exhibiting complete silencing. This partial effect is also observed at the level of target mRNA abundance in that a pooled population of treated animals might exhibit at most a fourfold reduction in mRNA levels. Thus, treated individuals have a spectrum of RNAi-induced phenotypes, which makes interpretation of gene function somewhat difficult. Moreover, there is frequently a variation in the strength of RNAi effects between different transformant lines carrying the same IR transgene. This is likely due to influence of nearby chromosomal modulation of transgene expression that depends on the point of transgene insertion. Since RNAi silencing is not complete, weak or strong IR expression translates to a corresponding weak or strong silencing effect. A second problem with this first-generation RNAi vector has been the variable success in cloning IRs from different genes. Some IRs are easily cloned even into *sbcBC*<sup>+</sup> bacterial strains. However, some IRs have proved recalcitrant to cloning in any strain or any plasmid. To date, we have been unable to predict which sequences will produce problems when cloned as IRs. This makes transgene production a somewhat empirical trial-by-error process.

### 3. Transgenic RNAi with inverted exon repeats

The problems with the first-generation vector inspired us to devise a new approach to produce hairpin

RNA in vivo. It was reported that intron-spliced hairpin RNA can induce gene silencing in plants more efficiently than standard hairpin-loop RNA [15]. In a sense, the inverted repeats are structural and functional exons. The nonpalindromic intron sequence may also provide stability to the DNA construct with inverted repeats in bacteria. This led us to test an RNAi construct containing inverted repeats separated by an intron sequence, from which loopless hairpin dsRNA is predicted to be produced following splicing in *Drosophila* (Fig. 2). As a proof-of-principle demonstration of its effectiveness, we decided to test the approach on silencing the *white* gene.

The *Drosophila white* gene encodes an ABC transporter involved in localizing pigments to eye pigment granules [16]. The *white* gene was chosen because expression can be easily monitored phenotypically by changes in eye color. A *white*<sup>+</sup> eye is dark red in color while the eye of a null *white* mutant is completely white in color (Fig. 3). Since cloned variants of *white* are routinely used in *Drosophila* transformation vectors as the selectable marker for transformation of *white* mutant flies, we adopted an opposing transformation strategy. That is, we constructed a transformation vector with a *white* IR but no independent marker gene for selecting transformants. We then transformed *white*<sup>+</sup> flies with the vector and selected transformants that had a *white* loss-of-function phenotype. If an inserted *white* IR transgene successfully silenced its endogenous target gene, the transformant would be white-eyed.

The 74-nucleotide second intron of the *white* gene bears all features of a consensus *Drosophila* intron, and it was found to efficiently splice in *Drosophila* embryonic extracts in vitro [17,18]. Since *white* is normally not expressed in embryos, this result indicates that the intron can be spliced in heterologous tissues. Thus, we chose the second intron to separate inverted repeats of *white* coding sequence in our model transgene.

The 629-bp third exon of *white* was chosen to be the inverted sequence in the transgene that would mediate the RNAi effect. The third exon was amplified by PCR with unique *Pst*I and *Eco*RI sites, and it was ligated in inverted orientation upstream of a 703-bp fragment containing the *white* second intron and third exon (Fig. 2). The tail-to-tail repeat was placed into the pGMR transformation vector plasmid [19]. pGMR drives expression of transgenes specifically in the developing and adult compound eye by virtue of the eye-specific GMR promoter. This ensured that the *white* IR transgene would be expressed only in the same cells that normally express the endogenous target *white* gene. The *Drosophila* consensus sequence for a 5' splice site is AG|GTRAGT, where | designates the splice site and R indicates A or G [17]. It is noteworthy that the ligation between the two DNA fragments through the *Pst*I site does not change the consensus sequence required for

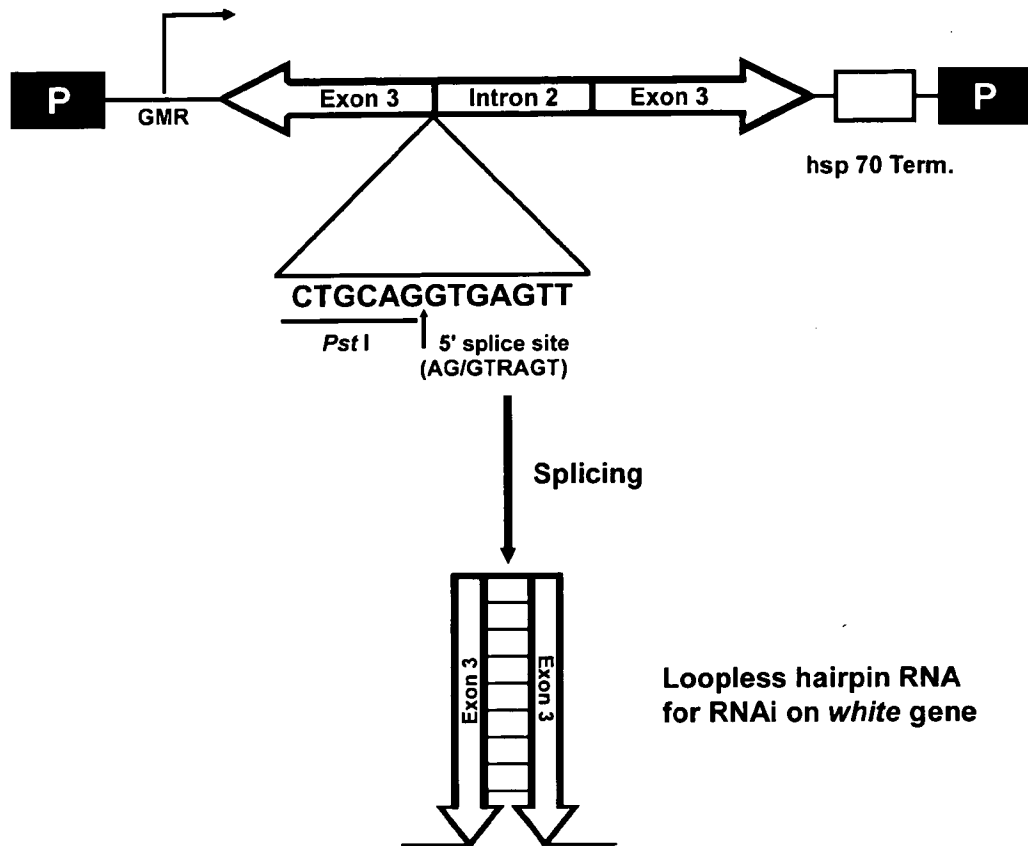


Fig. 2. Scheme for the generation of transgenic RNAi against the *Drosophila white* gene by intron-spliced hairpin RNA. Inverted repeats corresponding to the third exon of the *white* gene and separated by the second intron of the same gene were placed into the pGMR transformation vector. A fragment containing the *white* second intron and third exon was ligated to a fragment containing the inverted *white* third exon to generate a *Pst*I site at the point of ligation. Note that the *Pst*I site is coincident with the 5' splice site but does not disrupt sequences necessary for 5' splice site function. The *Drosophila* consensus sequence for a 5' splice site is shown in parentheses, in which / designates the splice site and R indicates A or G. The transgene is under transcriptional control of the GMR enhancer, which is exclusively active in the developing and adult retinal tissue that also expresses the endogenous *white* gene.

5' splice site recognition (Fig. 2). Since we intended to induce RNAi on the endogenous *Drosophila white* gene, an *Xho*I–*Nsi*I fragment corresponding to the pGMR *white* marker gene was deleted from pGMR.

Although we transformed ligation products including inverted repeats into the SURE strain (Stratagene) of *E. coli* to maximize the stability of the DNA, the repeats were also stable in a DH5 $\alpha$  strain, possibly as a result of the short *white* second intron sequence contributing to the stability of the inverted-repeat sequences. In contrast, attempts at cloning an IR of the *white* third exon separated by a *Sfi*I spacer into plasmids was repeatedly unsuccessful in a variety of host bacterial strains.

The pGMR-derived plasmid containing the DNA fragment for intron-spliced *white* hairpin RNA was introduced into the germ line of CantonS flies by P element transformation [14]. From approximately 1500 injected animals, eight independent transformant lines that exhibited a *white* loss-of-function phenotype were established. This transformation frequency is within an order of magnitude of the average transformation fre-

quency using a standard P element vector [14], which suggests that *white* RNAi from the IR transgene acts as a reliable marker for transformation. All eight transformant lines exhibited a yellow to pale-yellow eye color phenotype with one copy of the transgene (Fig. 3). Moreover, all individual flies from each line exhibited a uniform eye color phenotype, indicating strong penetrance and expressivity of the RNAi effect. Only females were compared to avoid any effect related to dosage compensation of the transgene. No additional or abnormal phenotypes were observed in silenced individuals, indicating that silencing was specific. The effect was stably maintained over each adult's lifetime, and silencing has been maintained over the many generations that these lines have so far been kept. Transformant adults bearing two copies of the transgene had an eye color indistinguishable from that of *white* null mutants (Fig. 3). Levels of *white* mRNA on a Northern blot were reduced in two transformant lines tested compared to wild type, to a degree consistent with their eye color phenotypes (data not shown). In conclusion, the

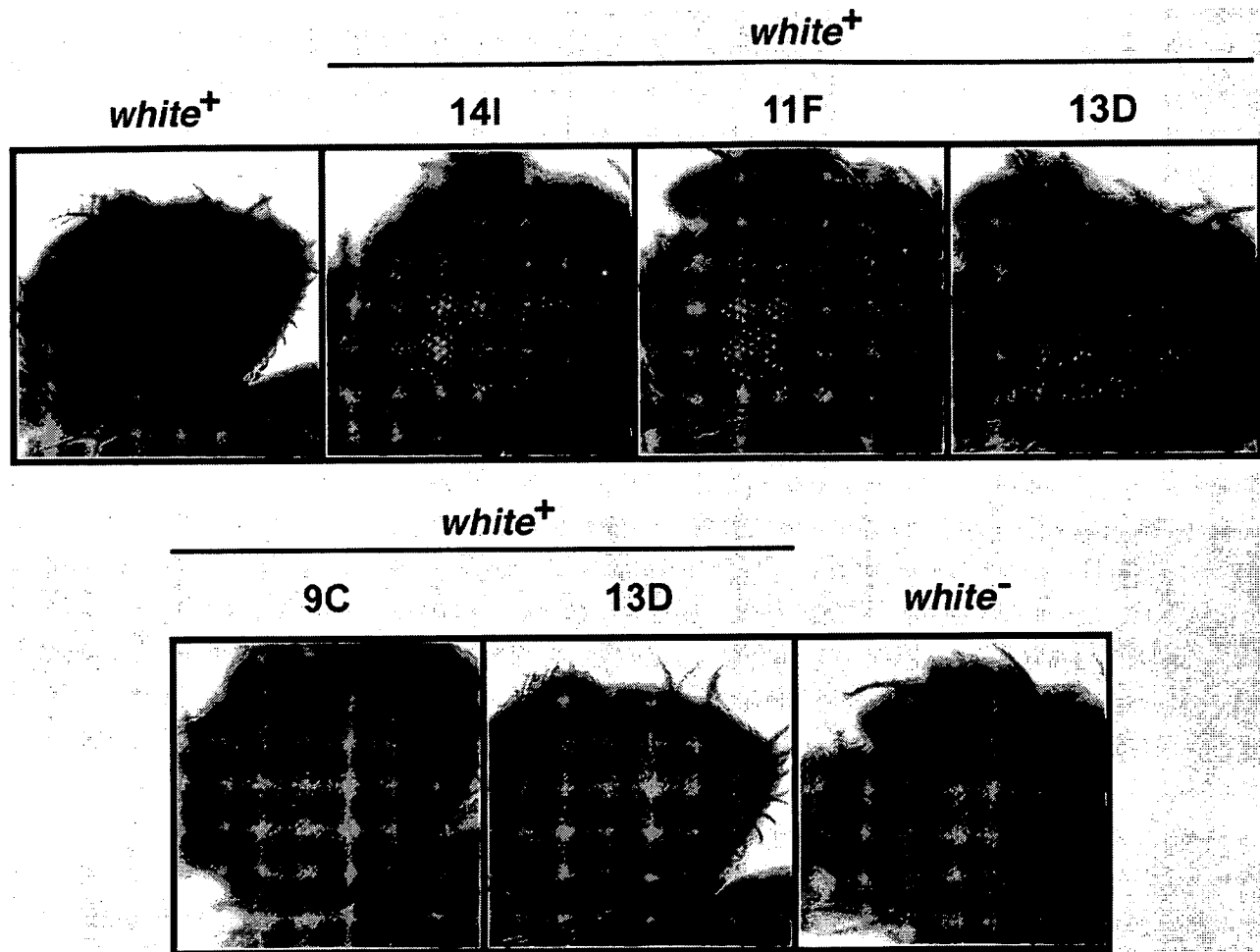


Fig. 3. Eye color phenotypes of female adults (3–5 days of age) that carry the *white* intron–hairpin GMR transgene. The top left shows a parental CantonS (wild-type *white*) fly. The lower right shows a *w<sup>1118</sup>* null mutant for the *white* gene. A P[*GMR-whiteIR*] transformant line designated 13D has the transgene on the X chromosome of the parental CantonS strain, whereas the 9C, 11F, and 14I lines have the transgene on the third chromosome of CantonS. The eye colors of transformants bearing a single copy of the transgene is shown in the top. The bottom shows eye colors of transformants bearing two copies of the transgene.

inverted exon repeat of *white* effectively silences the expression of the endogenous *white* gene in vivo.

#### 4. A modular and multipurpose transgenic RNAi vector

Since the RNAi construct bearing an intron strongly inhibited *white* gene expression, we adapted this method to create an all-purpose RNAi vector that employs spliced hairpin RNA. The vector is derived from the pUAST transformation plasmid. This then offers the advantages of the GAL4/UAS modular expression system, as outlined earlier. We constructed the vector (pWIZ, for white intron zipper) into which gene fragments can be subcloned upstream and downstream of the 74-nucleotide *white* intron (Fig. 4). The intron is flanked by *EcoRI*, *BglIII*, *NotI*, *XhoI*, *SpeI*, and *AvrII* sites on the 5' side and by *NheI*, *MluI*, and *XbaI* sites on the 3' side. The entire cassette is downstream of the UAS

enhancer–promoter and upstream of the SV40 transcription termination site. The *AvrII* and *NheI* sites in pWIZ conform to the consensus sequences for 5' and 3' splice sites, respectively. Thus, any DNA fragment inserted into the *AvrII* or *NheI* site is fully competent to be spliced as an exon. Moreover, the *SpeI*, *AvrII*, *NheI*, and *XbaI* sites are unique in pWIZ, providing convenient cloning sites for gene fragments.

To construct an IR transgene using pWIZ, a DNA fragment corresponding to the gene of interest is inserted twice into pWIZ, with inserts in opposite orientations on each side of the intron (Fig. 5). The simplest means to insert the DNA is as a PCR fragment. The system is designed so that a single PCR fragment derived from only two PCR primers can be inserted on each side of the intron. This is because *SpeI*, *AvrII*, *NheI*, and *XbaI* sites are all ligation-compatible with each other. Consequently, restriction sites compatible with *AvrII* and *NheI* sites should be placed in the PCR



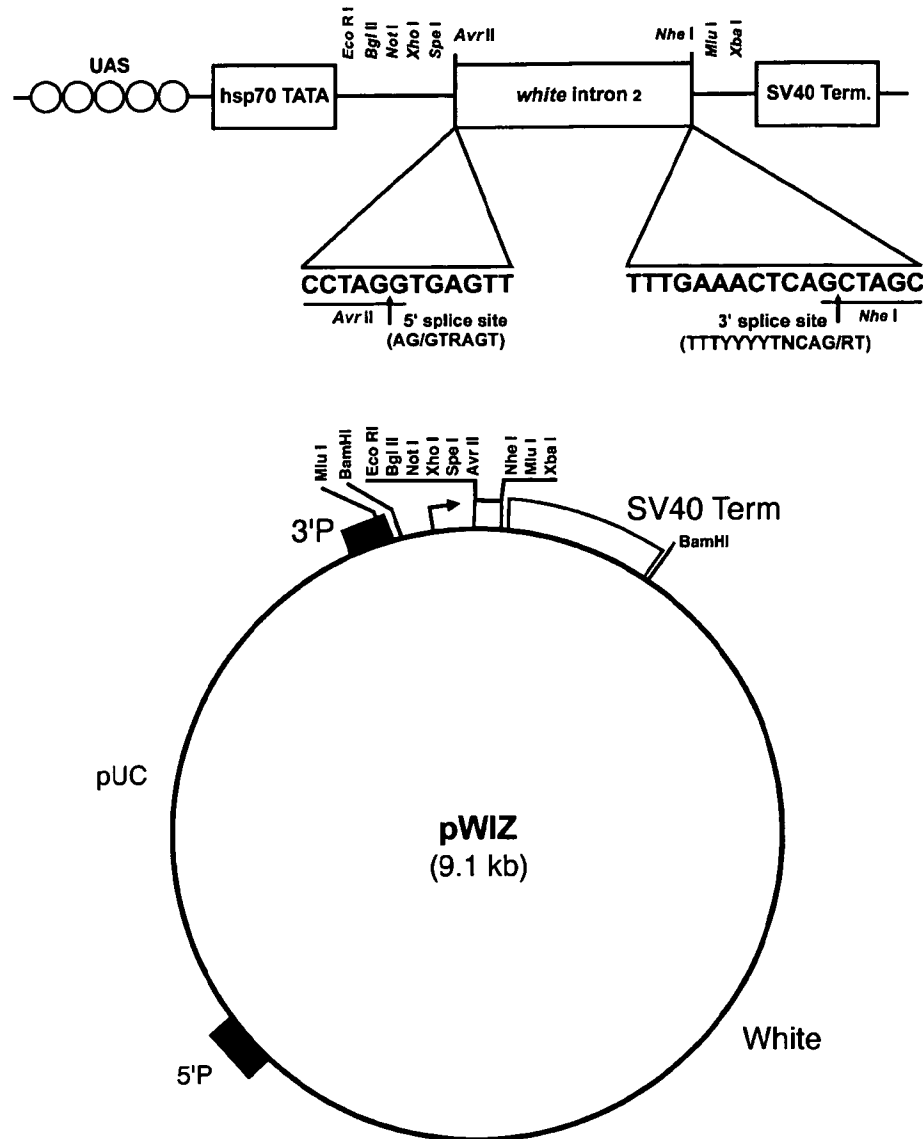


Fig. 4. Schematic representation of the pWIZ vector. The pWIZ vector was constructed by placing the 74-bp second intron of the *white* gene into the pUAST transformation vector [13]. The intron is flanked by unique *EcoRI*, *BglII*, *NotI*, *XhoI*, *SpeI*, and *AvrII* sites on the 5' side and *NheI* and *XbaI* sites on the 3' side to facilitate cloning. The sequences at the junction of the 5' and 3' splice sites in the vector are highlighted, and arrows indicate the 5' and 3' splice sites. The consensus sequences for 5' and 3' splicing are shown in parentheses: /, the splice site; R, purine; Y, pyrimidine; N, any base. Below is shown a restriction map of the pWIZ plasmid.

primers at their 5' ends. The resulting PCR product will then have *AvrII*- and *NheI*-compatible ends after appropriate restriction digestion. The RNAi construct is made by two sequential insertions of the same PCR product into the *AvrII* and *NheI* sites of pWIZ (Fig. 5). For efficient digestion, we add an extra 4 nucleotides to the 5' side of each primer restriction site. The size of amplified DNA ranges from 500 to 700 bp. Most important, we ensure that the sequence does not contain any internal restriction sites used in the PCR primers, nor should the fragment have sequences in either sense or antisense orientation that match either 5' or 3' consensus splice sites. This latter aspect is important to

prevent cryptic splicing from disrupting hairpin RNA formation. The PCR product is digested with the appropriate enzyme and ligated into the *AvrII* site of pWIZ. After a clone with the desired orientation of the insert is selected by restriction mapping, the same PCR product is ligated into the *NheI* site of the pWIZ derivative, and recombinants with the insert in opposite orientation to the first are screened and selected.

We have made five transgenic RNAi constructs for genes under study in our laboratory using the pWIZ vector. All of these constructs are stable as inverted repeats in *E. coli* strains such as SURE cells. Moreover, they have been introduced into the *Drosophila* genome

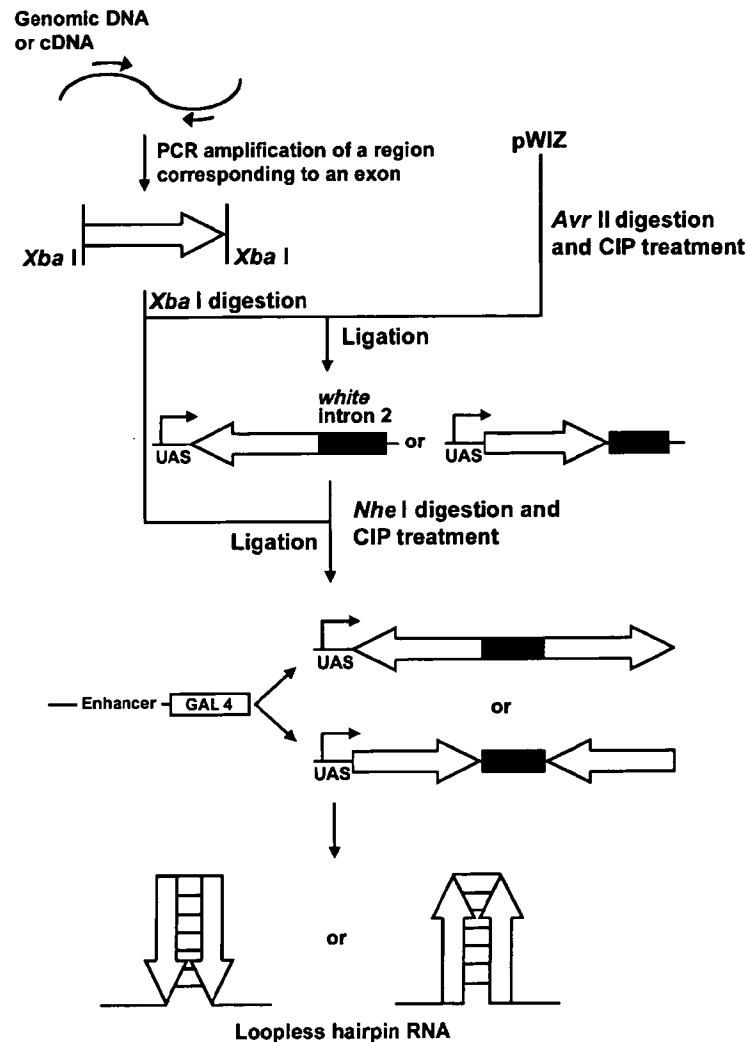


Fig. 5. A typical procedure for making a RNAi construct using the pWIZ vector. A DNA fragment corresponding to a *Drosophila* target gene is amplified by PCR. A restriction site (*SpeI*, *AvrII*, *NheI*, or *XbaI*) compatible with *AvrII* and *NheI* should be present at the 5' end of each PCR primer. The fragment can correspond to part of an exon, a complete exon, or several contiguous exons. Several criteria should be used in choosing the fragment: it should be 500 to 700 bp in length, it should not have internal restriction sites corresponding to the PCR primer sites, and it should not have sequences in either sense or antisense orientation that match a 5' or 3' consensus splice site. This latter aspect is critical to prevent cryptic splice sites from disrupting proper splicing of the hairpin RNA. As shown, an *XbaI* site is generated at each end of a PCR-amplified exon for cloning the PCR product. The PCR product is inserted twice by two ligation steps into the *AvrII* and *NheI* sites of pWIZ. CIP, calf intestinal phosphatase used to dephosphorylate the 5' ends of vector DNA prior to ligation. Recombinants are selected in the desired orientation, such that after the second ligation step, inserts are in opposite orientation on either side of the *white* intron. IRs that are head-head or tail-tail repeats might be used depending upon whether cryptic splice sites are present in the constructs. Transformation follows to generate stable transgenic lines carrying the WIZ gene. Upon mating of transgenic animals harboring the WIZ gene with animals carrying tissue- or cell-specific GAL4 drivers, the F1 progeny produce loopless hairpin RNA. This induces RNAi against target genes in tissue- and cell-specific patterns.

by P element transformation, and all constructs transformed efficiently to give stable lines.

## 5. Concluding remarks

In conclusion, we have developed transgenic RNAi in *Drosophila* that can be applied to many developmental and physiological processes. Hairpin RNA produced from a transgene composed of inverted repeats can spe-

cifically silence gene expression in *Drosophila*. The presence of a spacer between the inverted repeats makes for easier cloning but is offset by a weaker silencing activity in vivo [8,20]. In plants, using a functional intron as the spacer between inverted repeats strongly enhanced silencing activity of the RNAi transgene [15]. We have shown that using a functional intron as a spacer between inverted repeats produces strong and uniform RNAi silencing in *Drosophila*. A similar observation has been recently noted in *Drosophila* when inverted repeats

composed of cDNA–genomic DNA hybrids are separated by functional introns [21]. We have also described a multifunctional RNAi transformation vector (pWIZ) containing an intron spacer that makes RNAi simple to perform for the following reasons. A single PCR fragment of a gene is sufficient to construct a targeting vector; the inverted repeat sequence need not have splice sites present since they are provided by pWIZ; splice sites are preserved when the repeat fragments are inserted; the intron spacer provides stability to the inverted repeats when the plasmid is replicated in *E. coli*. Once the vector is transformed into *Drosophila*, it is conditionally quiescent until crossed with GAL4-expressing lines. Many useful GAL4-expressing lines are available, making the RNAi approach adaptable for most studies of *Drosophila*. This method is likely to be very useful for analyzing the function of the many *Drosophila* genes for which no loss-of-function mutations are available. Finally, the method provides a powerful tool to create loss-of-function phenotypes in a manner conditional for particular tissues and developmental times.

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# Exhibit 17

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## The Cotton *ACTIN1* Gene Is Functionally Expressed in Fibers and Participates in Fiber Elongation

Xue-Bao Li,<sup>a,b</sup> Xiao-Ping Fan,<sup>b</sup> Xiu-Lan Wang,<sup>a</sup> Lin Cai,<sup>b</sup> and Wei-Cai Yang<sup>b,c,1</sup>

<sup>a</sup> College of Life Sciences, Central China Normal University, Wuhan 430079, China

<sup>b</sup> Temasek Life Sciences Laboratory, National University of Singapore, Singapore 117604

<sup>c</sup> Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100080, China

Single-celled cotton fiber (*Gossypium hirsutum*) provides a unique experimental system to study cell elongation. To investigate the role of the actin cytoskeleton during fiber development, 15 *G. hirsutum* *ACTIN* (*GhACT*) cDNA clones were characterized. RNA gel blot and real-time RT-PCR analysis revealed that *GhACT* genes are differentially expressed in different tissues and can be classified into four groups. One group, represented by *GhACT1*, is expressed predominantly in fiber cells and was studied in detail. A 0.8-kb *GhACT1* promoter sufficient to confirm its fiber-specific expression was identified. RNA interference of *GhACT1* caused significant reduction of its mRNA and protein levels and disrupted the actin cytoskeleton network in fibers. No defined actin network was observed in these fibers and, consequently, fiber elongation was inhibited. Our results suggested that *GhACT1* plays an important role in fiber elongation but not fiber initiation.

### INTRODUCTION

Actin cytoskeleton plays an important role in cell morphogenesis in plants as demonstrated by pharmacological, biochemical, and genetic studies (Kost and Chua, 2002; Mathur and Hülskamp, 2002). The actin cytoskeleton may be involved in the transportation of organelles and vesicles carrying membranes and cell wall components to the site of cell growth as in root hairs, trichome cells, and pollen tubes. Therefore, the actin cytoskeleton is essential for cell elongation and tip growth. Disruption of the actin cytoskeleton during trichome development by actin-interacting drugs resulted in randomly distorted trichomes with unextended branches (Mathur et al., 1999; Szymanski et al., 1999). Similarly, inhibition of F-actin elongation blocked the initiation of polar growth and elongation of root hairs (Miller et al., 1999). Furthermore, reduction in actin arrays resulted in dramatic reduction of root hair length and caused severe bulges in the *actin2* (*act2*) mutant and serious retardation of root growth in the *act7* mutant in *Arabidopsis thaliana* (Gilliland et al., 2002, 2003). Misexpression of the reproductive *ACT11* gene in vegetative tissues of *Arabidopsis* altered morphology of most organs in plants because of its effects on the proportion of different actin isoforms (Kandasamy et al., 2002). In polarized elongating cell types, such as root hairs and trichomes, it is believed that long F-actin cables oriented longitudinally throughout the shank and

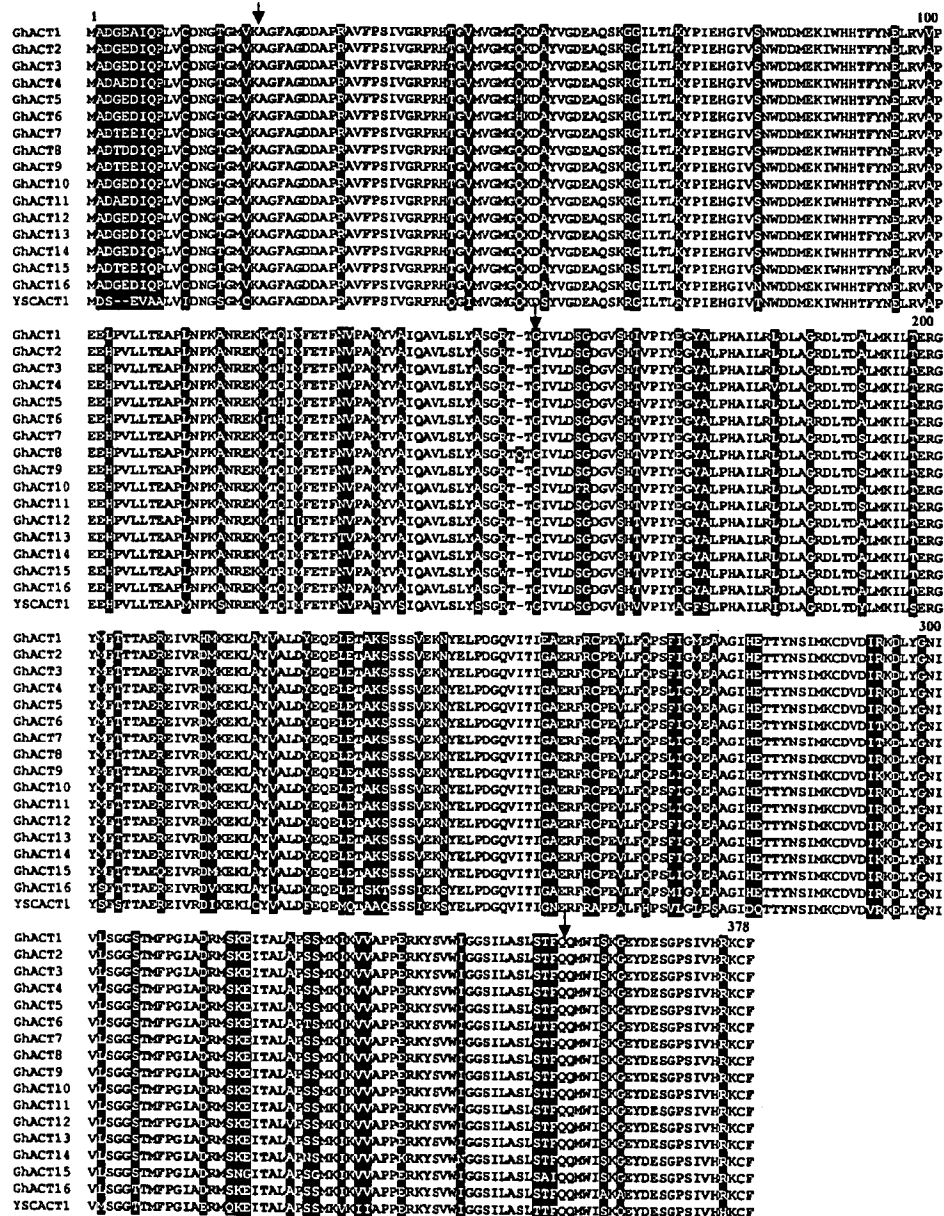
subapical, and net-axially aligned fine F-actins are essential for the intracellular trafficking of organelles and secretory vesicles to the growing apical region to deliver new membranous and cell wall materials (Mathur et al., 1999; Miller et al., 1999; Szymanski et al., 1999; Baluska et al., 2000; Hepler et al., 2001; Chueng et al., 2002). The unstable dynamic F-actin cytoskeleton also plays a role in localized expansion of root hairs and trichome cells (Ketelaar et al., 2003; Mathur et al., 2003a).

The actin cytoskeleton controls polar cell growth through its interaction with several actin binding proteins, such as actin depolarizing factor (Dong et al., 2001; Chen et al., 2002), profilin (Clarke et al., 1998), Rho family GTPase (Yang, 1998; Chueng et al., 2002; Fu et al., 2002), and the calcium signaling pathway (Malhó, 1998; Franklin-Tong, 1999; Li et al., 1999). The effective regulation of actin turnover by actin regulators may be critical for pollen tube growth (Chen et al., 2002, 2003) and for polar cell expansion in cell types other than root hair and trichome (Fu et al., 2002). Recent studies showed genetically that the actin cytoskeleton by interacting with the ARP2/ARP3 complex plays a pivotal role in controlling cell shape of trichome cells and several other cell types in *Arabidopsis* (Mathur et al., 2003a, 2003b). In cotton (*Gossypium hirsutum*), F-actin has been implicated in regulating microtubule orientation during fiber development shown by in vitro drug studies (Seagull, 1990). However, the role of the actin cytoskeleton in cotton fiber cell development remains largely unknown.

Actins in plants are encoded by a multigene family that comprises dozens or even hundreds of actin genes. In *Arabidopsis*, the actin gene family contains 10 distinct members, of which eight are functional genes and two are pseudogenes (McDowell et al., 1996). In other plant species, the actin gene family also appears to have dozens of members (Baird and Meagher, 1987; Thangavelu et al., 1993; Meagher and Williamson, 1994). Studies on actin sequences revealed that structural and

<sup>1</sup>To whom correspondence should be addressed. E-mail wcyang@genetics.ac.cn; fax 86-10-62551272.

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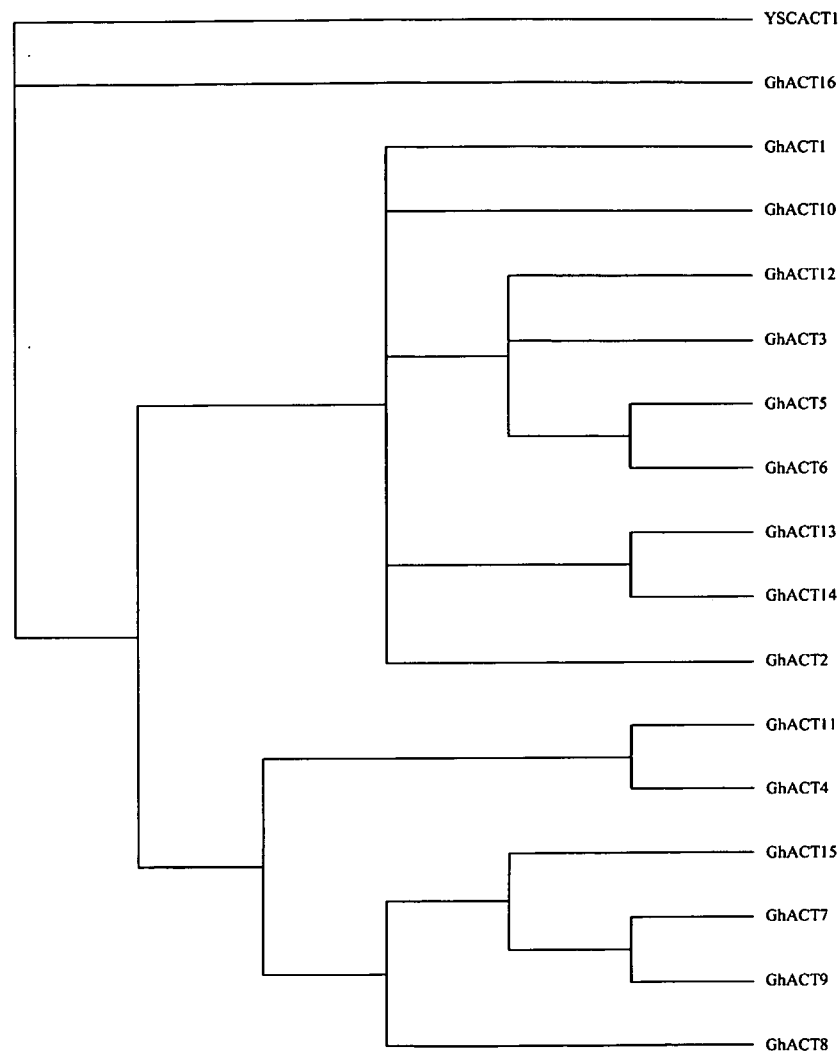


**Figure 1.** Comparison of the Predicted Amino Acid Sequences of Cotton *GhACT* Genes.

Multiple alignment of amino acid sequences of 16 cotton *GhACT* genes and yeast *YSCACT1*. Amino acid substitutions are highlighted in black. Arrows indicate the positions of the three introns in cotton *GhACT* genes. *GhACT1* to *GhACT15* were from this work; *GhACT16* is a putative actin derived from a genomic sequence in GenBank (accession number AF059484).

functional divergence occurred within the gene family during evolution (McDowell et al., 1996; Meagher et al., 1999a). Members of the actin gene family are divergent and differentially expressed during plant development. Arabidopsis contains two major actin gene classes: a vegetative class that is expressed predominantly in leaves, stems, roots, petals, and sepals and

a reproductive class that is strongly expressed in pollens, ovules, and embryonic tissues (McDowell et al., 1996; Kandasamy et al., 1999). The soybean (*Glycine max*) actin gene family includes at least three divergent classes:  $\mu$ -,  $\kappa$ -, and  $\lambda$ -actin. The  $\mu$ -actin transcripts are differentially accumulated in leaves, roots, and hypocotyls. The  $\kappa$ - and  $\lambda$ -actin proteins are preferentially localized



**Figure 2.** Phylogenetic Relationships of Cotton Actins.

The rooted gene tree shown is based on majority-rule consensus from 500 bootstrap replicates and resulted from heuristic searching in PAUP 4.0, based on amino acid sequences of the *GhACT* genes. Cotton GhACT1 to GhACT15 actins were from this work; GhACT16 is a putative actin derived from a genomic sequence in GenBank (accession number AF059484); YSCACT1 is a yeast actin (accession number L00026) used as an outgroup.

in roots (McLean et al., 1990). In other plant species, such as rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*), actin genes also appear to be expressed in a tissue-specific manner (McElroy et al., 1990; Thangavelu et al., 1993). Although actin genes in a few plant species such as *Arabidopsis* have been well characterized, our knowledge of cotton actin genes, especially its role in fiber development, needs to be explored.

Cotton fibers, as a premier natural fiber and extensively used in the textile industry, are derived from epidermal cells of the reproductive organ, the ovule. Approximately 30% of the ovule epidermal cells elongate and develop into single-celled fibers at anthesis. Each fiber is perhaps the longest single cell in higher plants. Its elongation rate and the final length attained are far

above that of common plant cells (Cosgrove, 1997). Fiber development is a highly regulated process involving four sequential stages: fiber initiation, primary cell wall formation, secondary cell wall formation, and maturation (Basra and Malik, 1984). Thus, the cotton fiber represents a unique experimental system for studying the control of cell elongation without the complication of cell division and multicellular development (Ruan et al., 2001). The study on fiber development not only provides the basic understanding of cell differentiation and elongation, but also identifies potential target genes for genetic manipulation of cotton fiber. Here, we reported the identification and characterization of the actin gene family in cotton and explored its role in fiber development using RNA interference (RNAi) technology.

**Table 1.** Primers Used in Gene-Specific RT-PCR of *GhACT* Genes

Genes	Primers
<i>GhACT1</i>	5'-CCCTTGAATATTAATAAATAAAAAATA-3' 5'-TTGTGCTCAGTGGGGGTTCAACC-3'
<i>GhACT2</i>	5'-TGCCCCGAAGTCTCTTCCAG-3' 5'-ATTTTCCCAGAAGTTTGACCGCGC-3'
<i>GhACT3</i>	5'-CCCTTGAATATTAATAAATAAGCAC-3' 5'-TTGTGCTCAGTGGGGGTTCAACT-3'
<i>GhACT4</i>	5'-GGGGGAGCCTTGAATATGAAATTG-3' 5'-TTGTGCTCAGTGGGGGTTCAACC-3'
<i>GhACT5</i>	5'-ATTTTCCCAGAAGTTTGACCGCGC-3' 5'-TGCCCCGAAGTCTCTTCCAA-3'
<i>GhACT7</i>	5'-TTAAAGAAAATAAGAAATAAGCATCA-3' 5'-GTATGCCAGTGGTCCGACGACA-3'
<i>GhACT8</i>	5'-TTAAAGAAAATAAGAAATAAGCATCA-3' 5'-GTATGCCAGTGGTCCGACGAG-3'
<i>GhACT9</i>	5'-ATCTTCAACATAAAAGATCATCCCACT-3' 5'-GATCTATCTTGGCATCACTCAGCA-3'
<i>GhACT10</i>	5'-AACCAGATATTAATATAATTTCCGTAG-3' 5'-GGGAAATTGTCCGTGACATGAAG-3'
<i>GhACT11</i>	5'-ACAATAGCTATTGACATTAATGTTTGC-3' 5'-TTGTGCTCAGTGGGGGTTCAACT-3'
<i>GhACT12</i>	5'-AACCAGATATTAATATAATTTCCGTAG-3' 5'-GGGAAATTGTCCGTGACATGAAA-3'
<i>GhACT13</i>	5'-CCCTTGAATATTAATAAATAAGCAC-3' 5'-TTGTGCTCAGTGGGGGTTCAACC-3'
<i>GhACT14</i>	5'-AACCAGATATTAATATAATTTCCGTAA-3' 5'-ATTGGAGCTGAGAGATTCCGTTG-3'
<i>GhACT15</i>	5'-ATCTTCAACATAAAAGATCATCCCACT-3' 5'-GATCTATCTTGGCATCACTCAGCG-3'
<i>GhUBI</i>	5'-CTGAATCTTCGCTTTCACGTTATC-3' 5'-GGGATGCAATCTTCGTGAAAC-3'

The efficiency of each primer pair was detected using *GhACT* cDNA clones as standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair.

## RESULTS

### Isolation and Characterization of *GhACT* cDNAs

To isolate genes involved in cotton fiber development, we have randomly sequenced >300 cDNA clones from a fiber cDNA library (Li et al., 2002). Clones, including an actin cDNA, likely involved in cell elongation were chosen for further study. Using the actin cDNA clone as probe, we further isolated 15 unique actin cDNAs (designated *GhACT* genes; accession numbers in GenBank, AY305723 to AY305737) from a cotton cDNA library. Sequence analysis predicted that all *GhACT* genes, except *GhACT8*, encode a 377-amino acid polypeptide. The *GhACT8* encodes an actin containing 378 amino acid residues with a Gln insertion at position 151 (Figure 1). The *GhACT* genes share high sequence homology at nucleotide level (70 to 97% identity) in the coding region and at the amino acid level (93 to 99% identity). There is only 1 to 7% substitution rate at amino acid level compared with each other (Figure 1). In total, 21 charged substitutions occurring at 14 charged positions were present in *GhACTs*. Among them, charged amino acids were exclusively

substituted with uncharged residues at six locations (Arg/Gly, Thr or Gln, Asp/Ala, Glu/Gly, His/Leu, or Lys/Trp) and were only substituted by a synonymous charged amino acid at other positions. The charged amino acids at residues 6 and 292 were substituted by either a charged amino acid or an uncharged residue (Figure 1), suggesting that these positions may not be important for actin structure. While at residue 123, both charged and polar uncharged amino acids were present in *GhACTs*. In addition, 11 uncharged amino acids at six positions were substituted by a charged residue. Often in this case, Gln was substituted by a His and Gly replaced by an Arg. Intriguingly, most nonsynonymous substitutions occur only in *GhACT1* protein. For example, positively charged amino acids were substituted by a nonpolar, uncharged amino acid at positions 64 and 103. On the other hand, nonpolar amino acids were replaced by positively charged and negatively charged polar residues at positions 121 and 253, respectively. At position 213, the negatively charged Asp was substituted by a positively charged His, suggesting that *GhACT1* may have a different structure and function than other *GhACT* variants.

Phylogenetic analysis on amino acid sequences showed that the 16 *GhACTs* available could be divided into nine subgroups (Figure 2). Among them, five subgroups contain only a single member, and the remaining four subgroups have two to four members. Each of *GhACT1*, *GhACT2*, *GhACT8*, *GhACT10*, and *GhACT16* forms an independent clade, suggesting that these *GhACTs* diverged early during evolution, whereas *GhACT3*, *GhACT5*, *GhACT6*, and *GhACT12* together form a single branch, indicating that divergence of these genes occurred relatively late.

### *GhACT* Genes Are Differentially Expressed in Different Organs

To identify *GhACT* genes that are preferentially expressed in cotton fibers, the expression patterns of 15 *GhACT* cDNA clones were analyzed by real-time quantitative SYBR-Green RT-PCR using gene-specific primers (Table 1) as described in Methods. The cotton polyubiquitin gene (*GhUBI*; X.B. Li and W.C. Yang, unpublished data) expressed equally in all tissue types with cycle threshold (Ct) values at  $17.52 \pm 0.35$  and was chosen as a standard control to normalize differences in RNA template concentrations. Five out of the fifteen *GhACT* genes are expressed at relatively high levels in fiber cells (Figure 3A). *GhACT2* is expressed at high levels in all tissues compared with other *GhACTs*, and its expression level reaches a relative value of 17 in fibers as compared with ~5 in other tissue types. For example, *GhACT2* expression in fibers is ~370-fold higher than *GhACT14*. *GhACT1* and *GhACT5* are strongly expressed in fiber and very low in leaf, stem, root, and anther, indicating that they are preferentially expressed in fiber cells. *GhACT4* and *GhACT11* also showed similar expression patterns as *GhACT1* and *GhACT5* in fiber and were moderately expressed in other tissues, whereas the transcripts of other *GhACT* genes are very low, as shown in the small values in the y axis. Overall, *GhACT3*, *GhACT9*, *GhACT10*, and *GhACT12* are expressed at least five-fold less in fibers compared with *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11*. By contrast, the expression of *GhACT7*,

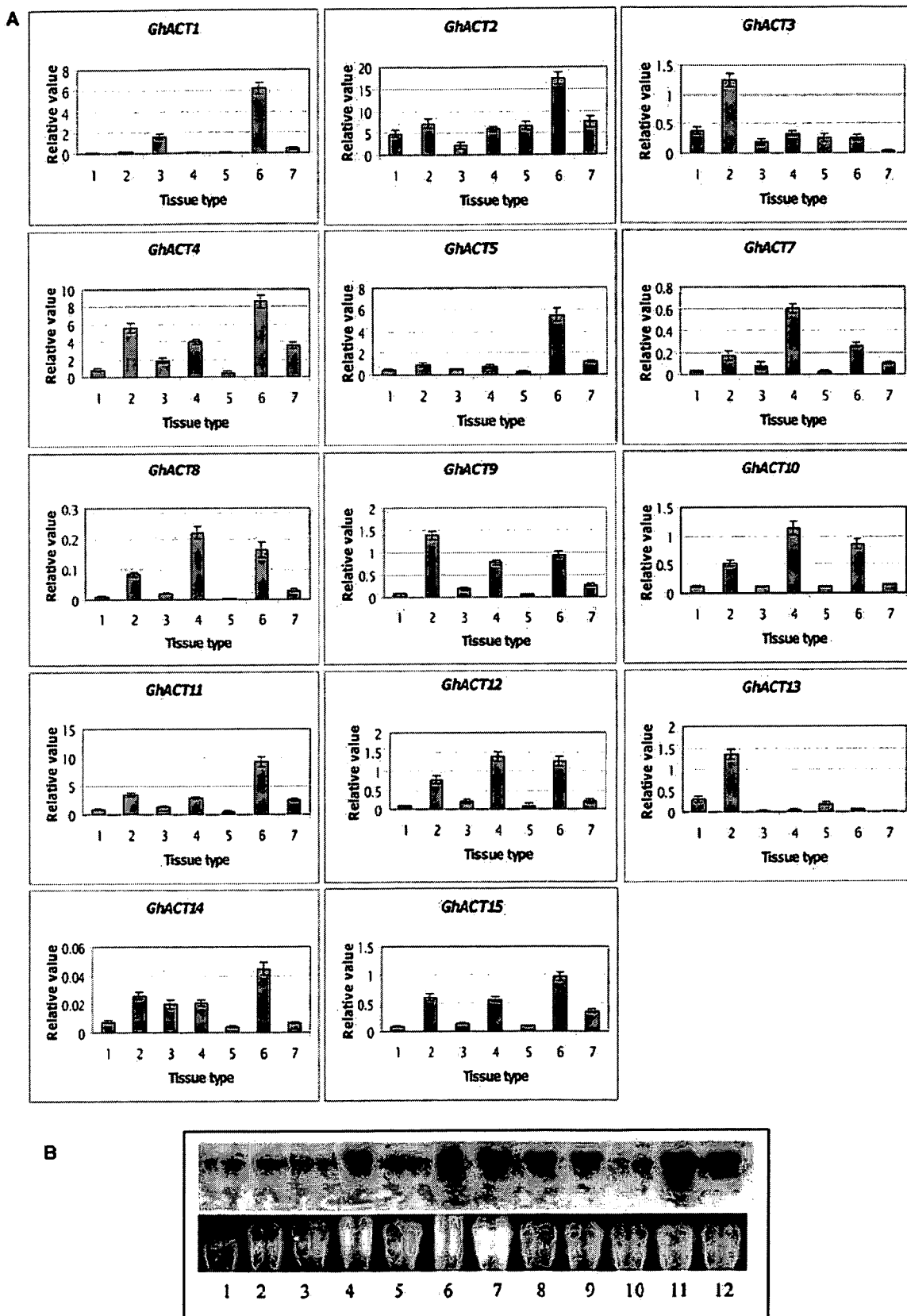


Figure 3. Analyses of Expression of *GhACT* Genes in Cotton Tissues.



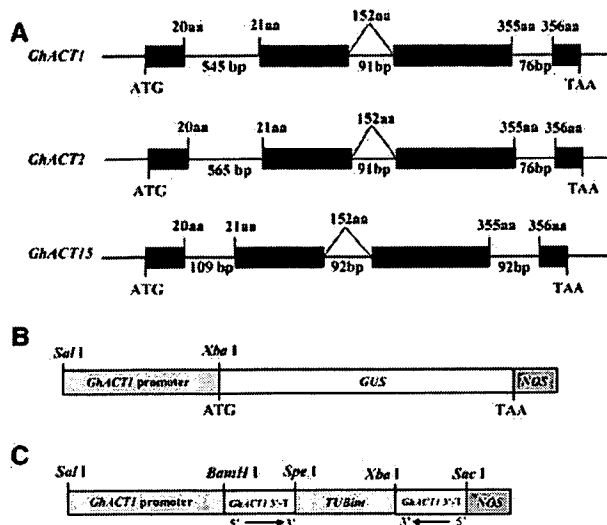
*GhACT8*, *GhACT14*, and *GhACT15* is extremely low if compared with *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11*. Moreover, *GhACT6* expression is not detectable in all the tissues examined. The results of the real-time RT-PCR revealed that the actin genes in cotton were differentially expressed, with *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11* being the predominant forms in fiber cells (Figure 3A).

RNA gel blot analysis, using the 3'-untranslated region (UTR) of *GhACT1* as a probe, further demonstrated that *GhACT1* accumulated at high level in fibers and at a relatively lower level in ovules. The level of *GhACT1* transcripts reached the highest level during 8 to 14 d postanthesis (DPA) and decreased gradually as the ovule developed. At 28 DPA, hardly any transcript was detected. No or very little transcripts were detected in anthers, petals, leaves, and roots (Figure 3B). A moderate level of *GhACT1* was detected in cotyledons. This result further confirmed that the *GhACT1* gene is preferentially expressed, especially in elongation phase in cotton fiber cells.

#### Isolation and Characterization of *GhACT* Genes

Five genomic DNA clones, representing *GhACT1*, *GhACT2*, and *GhACT15* (Figure 4A), were isolated from a cotton genomic library using *GhACT1* cDNA as probe. The isolated *GhACT1* gene is ~3.9 kb in length, including 1.6 kb of the 5' promoter region, 1.8 kb transcribed region, and 0.5 kb 3' downstream sequence. Sequence comparison between cDNA and genomic clones revealed that the three *GhACT* genes all contain four exons and three introns (Figure 4A). The three introns are located exactly at the same positions in all three genes: between amino acid residues 20 and 21, within residue 152, and between residues 355 and 356, respectively. The size and position of introns in *GhACT1* and *GhACT2* are almost identical (Figure 4A). Intron 1 is 545 and 565 bp in length in *GhACT1* and *GhACT2*, respectively, much longer than intron 2 and intron 3. By contrast, intron 1 in *GhACT15* is relatively short, with only 109 bp. The lengths of introns 2 and 3 are similar in all three genes. These data indicate that *GhACT* gene structure is quite conserved in cotton.

To determine the actin gene family copy numbers, cotton genomic DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I, and *Xba*I and subjected to DNA gel blot analysis. There was one major band and one to two weak bands when the 0.8-kb 5' noncoding region of *GhACT1* was used as a probe. The major band represents *GhACT1*, and the weaker bands most likely are due to cross-hybridization with other members of the actin gene family, though the 5' noncoding region was used as probe (Figure 5A). Furthermore, several bands were detected when using the more conserved exon 3 of *GhACT1* as a probe under highly



**Figure 4.** *GhACT* Gene Structure, *GhACT1::GUS*, and *GhACT1* RNAi Construction.

(A) Exons are denoted by black boxes. Introns, 5'-flanking region, and 3'-UTR are denoted by lines. The lengths of the introns in base pairs are indicated. The number at the boundaries of each exon indicates the codon at which the intron is located. The translation initiation and termination codons are shown. aa, amino acids.

(B) The length of the *GhACT1* promoter and cloning sites used for *GhACT1::GUS* fusion are shown.

(C) *GhACT1* RNAi construction.

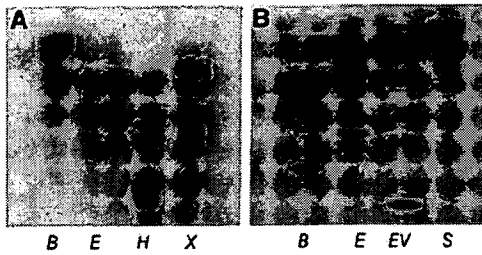
stringent conditions (Figure 5B). This suggested that there are at least four to eight members of the actin family that share a highly conserved coding region with *GhACT1*, and the remains may diverge earlier during the evolution of the cotton actin gene family.

#### The *GhACT1::β-Glucuronidase* Fusion Gene Is Predominantly Expressed in Cotton Fibers

To characterize the precise expression pattern of *GhACT* genes in cotton fibers, we chose *GhACT1* for further study because it represents *GhACTs* that are expressed preferentially in fibers (Figure 3A) among the three available genomic sequences. A 0.8-kb promoter region of *GhACT1* was subcloned upstream of the  $\beta$ -glucuronidase (*GUS*) reporter gene in pBI101 vector, giving rise to the *GhACT1::GUS* gene (Figure 4B). The *GhACT1::GUS* construct was introduced into cotton cultivar Coker312

**Figure 3.** (continued).

(A) Real-time RT-PCR analysis of expression of *GhACT* genes in cotton tissues. Relative value of *GhACT* gene expression in cotton tissues, including leaf (1), stem (2), cotyledon (3), root (4), anther (5), fiber (6), and petal (7), was shown as percentage of *GhUBI* expression activity (see Methods). (B) RNA gel blot analysis of *GhACT1* transcripts in cotton. Total RNA (20  $\mu$ g/lane) from petal (1), anther (2), leaves (3), cotyledon (4), root (5), ovule (6 to 10) at 4, 8, 14, 21, and 28 DPA, and fiber (11 and 12) at 8 and 14 DPA was fractionated on a 1.2% denaturing agarose gel and transferred onto a nylon membrane (see Methods). Top panel, autoradiograph of RNA hybridization; bottom panel, RNA gel before transfer to membrane showing equal loading of RNAs.



**Figure 5.** Genomic DNA Gel Blot Analysis of the *GhACT1* Gene.

Thirty micrograms of genomic DNA was digested with restriction enzymes as indicated and fractionated on a 0.8% agarose gel. DNA gel blots were hybridized with  $^{32}\text{P}$ -labeled *GhACT1* 5'-region gene-specific probe (0.8 kb) (A) and  $^{32}\text{P}$ -labeled *GhACT1* exon 3 probe (0.6 kb) (B). B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xba*I; EV, *Eco*RV; S, *Sac*I.

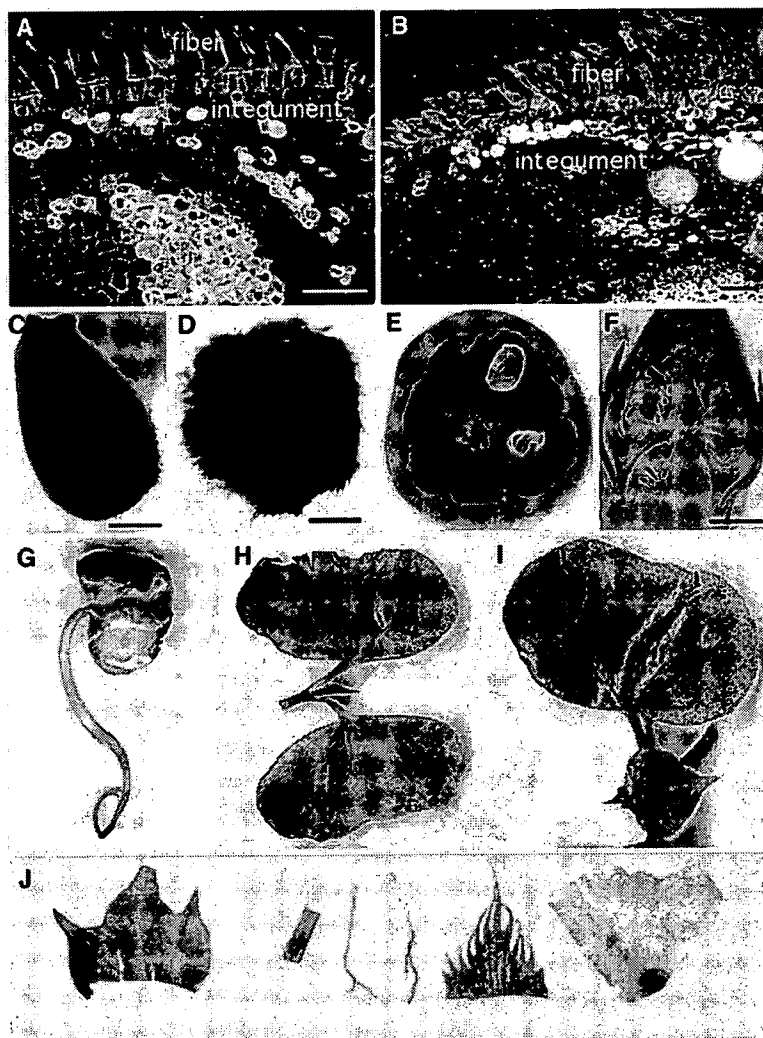
via *Agrobacterium tumefaciens*-mediated DNA transformation. A total of 230 transformed T0 plants from 21 independent calli were obtained and transplanted to soil for seeds. A total of 52 of 230 T0 transgenic plants were examined in detail for *GUS* expression patterns. In all of the 52 transgenic plants examined, strong *GUS* activity was detected only in young fibers (Figures 6A to 6E), whereas no or weak *GUS* staining was observed in ovules, anthers, petals, sepals, leaves, and roots, including their trichomes (Figures 6F and 6J). In comparison, plants transformed with the positive control pBI121 (35S::*GUS*) exhibited strong *GUS* activity in all tissues, and the nontransformed plants showed no *GUS* activity in fibers as well as in other tissues under the same staining conditions (data not shown). The same pattern of *GhACT1*::*GUS* expression was further confirmed in T1 and T2 transgenic plants. In addition, the *GhACT1*::*GUS* expression was observed at a moderate level in cotyledons of the germinating embryos at the first 1 to 2 d, when the root had just emerged from the embryo and the two cotyledons had not yet unfolded. In 3- to 4-d-old seedlings, moderate *GUS* activity was still observed in the cotyledon tissues (Figure 6G). Hypocotyls showed a low level of *GUS* activity in only one of the 21 independent transgenic lines examined. *GhACT1*::*GUS* expression was not detected in the roots of 3- to 8-d-old seedlings. Occasionally, weak expression was detected in the root tip in one transgenic line. As the seedling grew, *GUS* activity gradually decreased and finally disappeared in the cotyledons (Figures 6H and 6I). In 2-week-old seedlings, no significant *GUS* activity in the transgenic plants was detected. These results indicated that the 0.8-kb *GhACT1* promoter was sufficient to direct its fiber-specific expression and regulate its dynamic expression during cotton plant development.

#### Suppression of *GhACT1* Expression Dramatically Reduces Fiber Elongation

To study the role of actin cytoskeleton in fiber elongation, we chose a *GhACT1* gene that is expressed preferentially in fibers and less expressed in other tissues or organs (Figure 3A). Therefore, it was expected that knockdown of this gene would

have no or less effect on other tissues. Knockdown approaches using RNAi technology were employed. The 150-bp 3'-terminal fragment of *GhACT1* was constructed in the opposite direction with an intron from a cotton tubulin gene as a spacer (Li et al., 2002), then subcloned into pBI101 downstream of its own promoter (Figure 4C) and introduced into cotton cultivar Coker312 via *Agrobacterium*-mediated DNA transfer. Fourteen independent transgenic lines were regenerated. RNA gel blot analysis showed that the level of *GhACT1* mRNAs was reduced significantly down to very low level in fibers of the transgenic plants, using *GhACT1* 3'-UTR fragment as a probe (Figure 7A). To understand whether the reduced actin mRNAs also include other *GhACT* gene products, we further analyzed the expression levels of all the *GhACT* genes in fibers from RNAi transgenic plants by real-time quantitative SYBR-Green RT-PCR using gene-specific primers (Table 1). The results revealed that the expression of the *GhACT1* RNAi resulted in complete *GhACT1* silence in line T1 and ~10-fold reduction in lines T2, T3, and T4 (Figure 8). On the contrary, its impact on the expression of other *GhACT* genes was minor, with ~10% reduction (Figure 8). To confirm that the reduction in *GhACT1* mRNA also led to reduction at the actin protein level, protein gel blot analysis using actin antibody was performed. A strong band was detected in nontransgenic control fibers, whereas no or weak signals were detected in the transgenic lines (Figure 7B). This indicated that there was significant reduction in the actin proteins (mostly GhACT1) as a result of the reduction in *GhACT1* expression, and the remaining signals in the transgenic lines (Figure 7B, lanes 2 to 5) likely represented the other *GhACT* proteins expressed in fibers or residual GhACT1. These data suggest that GhACT1 is one of the dominant and functional actin isoforms in fibers.

All *GhACT1* RNAi transgenic plants showed a short-fiber phenotype (Figure 9) that cosegregated with the kanamycin selection marker (data not shown) and the reduction of actin protein levels, indicating that the phenotype was a result of the actin reduction caused by *GhACT1* silence. Fiber cells differentiate and rapidly emerge from the surface of the ovule at 0 to 1 DPA in wild-type plants (Figure 9A), whereas fibers in transgenic plants (Figure 9D) were much shorter. At 2 DPA, fiber cells in wild-type plants reached ~500  $\mu\text{m}$  long (Figure 9B), whereas transgenic fibers were only ~150 to 380  $\mu\text{m}$  in length (Figure 9E). Fiber length at 3 DPA in most transgenic plants (Figure 9F) was equal to fibers at 2 DPA in wild-type cotton (Figure 9B) and much shorter than fibers at 3 DPA in wild-type plants (Figure 9C). Measurement of fiber length showed that fiber elongation in transgenic plants was ~1.5- to 3-fold slower than that in wild-type plants (Figure 10), which correlated with the reduction of actin protein level (see Figure 7B). The results suggest that the reduction in total actins, including GhACT1, slowed down fiber elongation. Moreover, a portion of the ovules was sterile, and bolls in transgenic plants were smaller than those in the wild type after maturation, indicating that *GhACT1* RNAi also slightly affected pollination or seed development. However, all the transgenic lines were unaffected in vegetative growth and flower development. No inhibition on fiber initiation was observed in the *GhACT1* transgenic lines, suggesting the *GhACT1* gene is most likely not involved in fiber initiation but plays a role in fiber elongation.



**Figure 6.** Histochemical Localization of *GUS* Activity in Transgenic Cotton Plants Containing the *GhACT1::GUS* Fusion Genes.

(A) and (B) Dark-field micrographs of 5- $\mu$ m-thick cross sections of 1- to 2-DPA ovules. A high level of *GUS* activity (represented by pink dots) was only found in the fiber, and very weak *GUS* staining was seen in the inner cell layers. No *GUS* staining was detected in the epidermal atrichoblast and integument.

(C) to (J) Bright field of micrographs or photographs of ovules and other tissues/organs.

(C) and (D) *GUS* staining in ovules at 1 (C) and 2 (D) DPA. Strong *GUS* activity was observed in the fibers.

(E) A cross section of a transgenic cotton boll at 14 DPA. Strong *GUS* activity was detected in the developing fibers, and very weak *GUS* staining was seen in embryos.

(F) A longitudinal section of a transgenic flower bud before anthesis. Weak *GUS* staining was found in some pollen grains.

(G) to (I) *GUS* staining in transgenic seedlings.

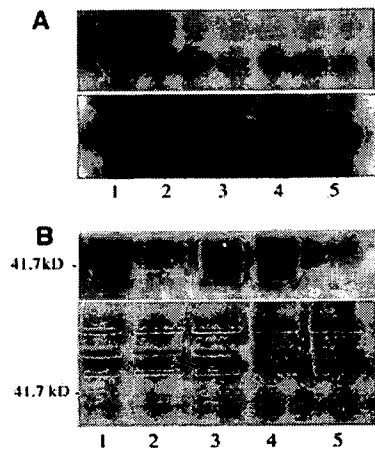
(G) Three-day-old seedling. *GUS* gene was expressed moderately in the cotyledons.

(H) Parts of a 7-d-old seedling. Weak *GUS* expression was found only in cotyledons.

(I) Parts of a 10-d-old seedling. *GUS* activity was very low in cotyledons, and no *GUS* expression was detected in other tissues, such as leaf and shoot apex.

(J) No *GUS* activity was detected in leaf, stem, root, sepal, and petal (from left to right) of transgenic cotton.

Bars = 80  $\mu$ m in (A) and (B), 1 mm in (C) and (D), and 2 mm in (F).



**Figure 7.** RNA Gel Blot and Protein Gel Blot Analyses of *GhACT1* Expression in RNAi Transgenic Fibers of Cotton.

**(A)** RNA gel blot analysis. Total RNAs from fibers at 10 DPA from a wild-type plant (1) and *GhACT1* RNAi transgenic lines (2 to 5) were fractionated on 1.2% denaturing agarose gel and transferred to a nylon membrane (see Methods). Top panel, autoradiograph of RNA gel blot hybridized with  $^{32}\text{P}$ -dCTP-labeled *GhACT1* probe; bottom panel, autoradiograph of the same RNA gel blot hybridized with  $^{32}\text{P}$ -dCTP-labeled 18S RNA probe (control) showing equal loading of RNAs.

**(B)** Protein gel blot analysis. Total soluble proteins of fibers at 10 DPA from a wild-type plant (1) and *GhACT1* RNAi transgenic lines (2 to 5) were separated by electrophoresis in a 12% SDS-PAGE gel. The protein gel blot was stained with anti-actin antibody (top panel) and Coomassie blue (bottom panel).

#### Impact of *GhACT1* Suppression on the Actin Cytoskeleton in Fiber Cells

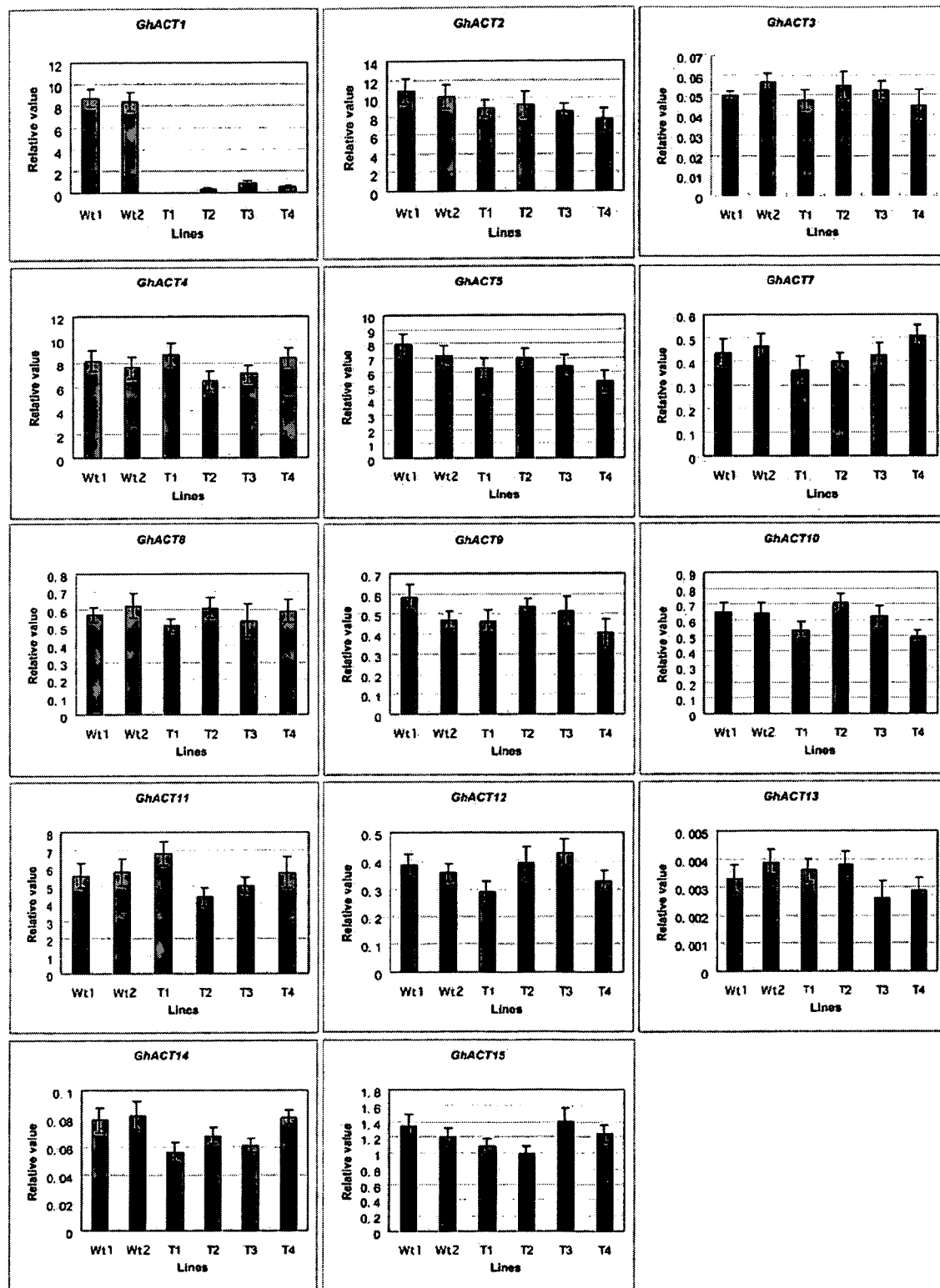
To investigate if changes in the actin cytoskeleton in *GhACT1* RNAi transgenic fibers occurred, we studied actin cytoskeleton in fiber cells using rhodamine-phalloidin staining for F-actin. During fiber cell elongation in wild-type plants, F-actin exhibited a complicated net-like structure from thin filaments to thick and longitudinally extending cables. At the early stage of fiber elongation, actin filaments were organized into arrays parallel to the growing axis and extended into the tip of the fiber cells (Figure 9G). With further elongation of fiber cells, the actin cytoskeleton was comprised of relatively thin arrays and thick cables along the long axis of the fiber (Figure 9H). The F-actin cytoskeleton displayed an increasingly complicated network consisting predominantly of thick and longitudinally long cables (Figure 9I). By contrast, actin filaments in transgenic fibers were obviously reduced in the number of filaments, and a more random array was observed (Figure 9J). During further development of the fibers, they were less bundled into arrays and cables (Figure 9K, compare with Figure 9H) and exhibited a defective F-actin organization (Figures 9K and 9L). As a result of RNAi of *GhACT1* expression in transgenic fibers, the devoid of the well-organized actin cytoskeleton consequently resulted in the reduction in fiber cell elongation, leading to the short-fiber phenotype. These data suggested that downregulation of

the *GhACT1* gene has an impact on actin cytoskeleton network in fiber cells.

## DISCUSSION

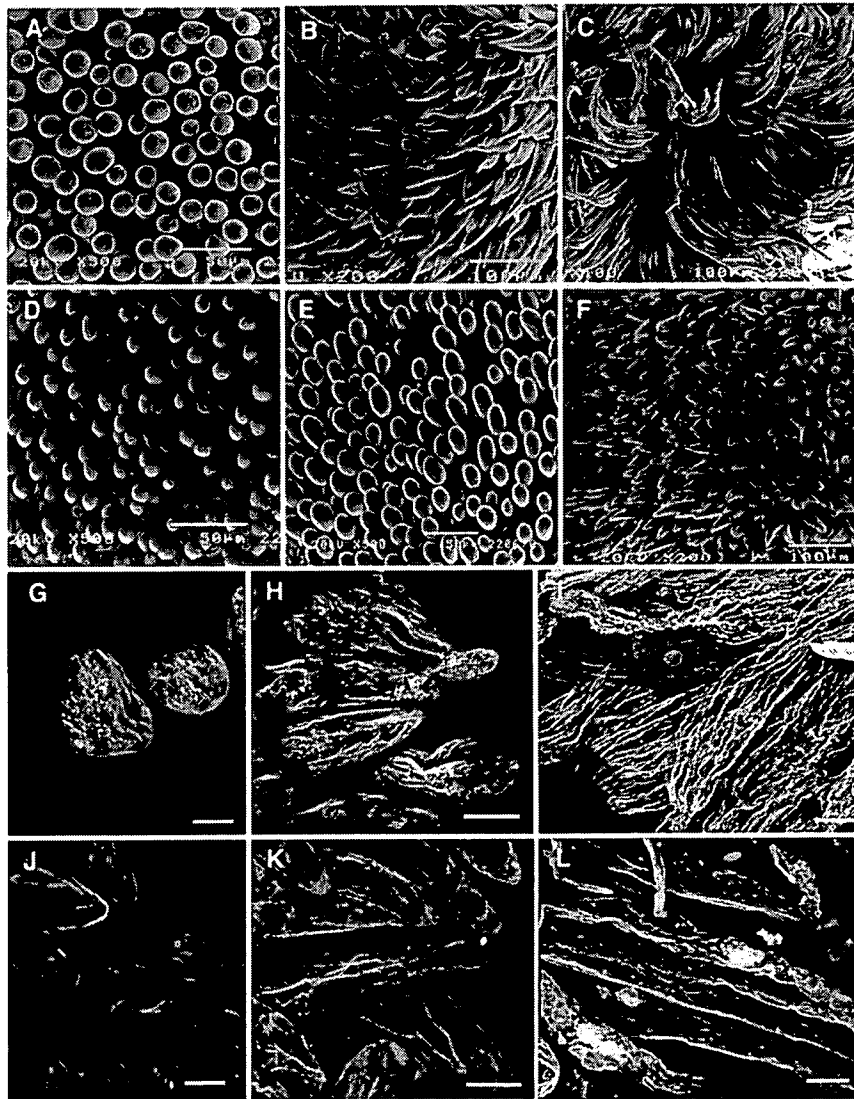
### Divergence of the Protein Structure of the GhACTs

Although plant actins are quite conserved, the divergence on protein structures occurred during evolution. In this study, the 16 cotton actins deduced from the isolated *GhACT* genes have diverged into nine subclasses compared with six subclasses in Arabidopsis (McDowell et al., 1996). Variation among GhACTs occurs more significantly than that found among the Arabidopsis actins. Figure 11 shows *GhACT1* protein structure, indicating that those significant substitutions on amino acids among the cotton actins may have an impact on their surface properties. The 14 positions where charged substitutions took place are found among the GhACTs, whereas only nine such positions appear among Arabidopsis actins (McDowell et al., 1996). At these positions, unlike Arabidopsis actins, only six positions were conservative substitutions, and the other positions showed nonconservative interchanges, whereas human actins contain only conservative substitutions. The nonconservative replacements of charged residues located on several surfaces of the actin molecule (Figure 11) may be involved in functional non-equivalency of actin isoforms as actin monomers polymerize from G-actin to F-actin and alter actin-actin or actin-actin binding protein interaction. For example, the uncharged polar Gln51 just adjacent to the DNase I binding loop involved in intermonomer interactions within the filament (Holmes et al., 1990) is replaced by a positively charged His in *GhACT5* and *GhACT6*. Because actin subdomain 2, in particular the DNase I binding loop, is directly involved in conformational changes (Otterbein et al., 2001), it is likely that this nonsynonymous substitution will have an impact on *GhACT5* and *GhACT6* structure and function. Recent genetic studies in Arabidopsis clearly showed that substitutions in AtACT2 have dramatic impact on its functions in root hair development (Gilliland et al., 2002; Ringli et al., 2002; Diet et al., 2004). Missense mutation in *der1* mutants (Ala183Val in *der1-1*, Arg97His in *der1-2*, and Arg97Cys in *der1-3*) all caused deformed root hairs. Furthermore, Glu356Stop in *enl2* enhances *der1* phenotypically (Diet et al., 2004), indicating that C-terminal residues are not absolutely required for its function. On the contrary, the conservative substitutions in the N-terminal peptide of Arabidopsis actins may affect polymerization and myosin binding (McDowell et al., 1996). Either a charged (His or Arg) or an uncharged polar amino acid (Gln) at position 123 of cotton actins may suggest that Gln and His are functionally interchangeable. Besides the charged substitutions, there were four positions where a noncharged Gly was substituted by a charged Glu or Arg seen in *GhACT1* (Gly<sub>253</sub> to Glu), *GhACT6* (Gly<sub>184</sub> to Arg), *GhACT8* (Gly<sub>158</sub> to Arg), and *GhACT14* (Gly<sub>297</sub> to Glu), respectively. These substitutions are not found in Arabidopsis actin genes (McDowell et al., 1996). It would be interesting to know whether these nonsynonymous substitutions have structural and functional impacts on transition from G-actin to F-actin.



**Figure 8.** Real-Time RT-PCR Analysis of *GhACT1* RNAi Expression in Transgenic Fibers.

Relative value of *GhACT* gene expression in 8-DPA fibers is shown as a percentage of *GhUBI* expression activity (see Methods). The *GhACT1* expression was significantly silenced by RNAi in the transgenic fibers, whereas the activities of the other *GhACT* genes were little affected in fibers of all the transgenic lines. Wt1 and Wt2, wild-type plants; T1 to T4, transgenic *GhACT1* RNAi lines.



**Figure 9.** Comparison of Fiber Growth Rate and F-Actin Organization in Fiber Cells between Transgenic *GhACT1* RNAi and Wild-Type Plants.

(A) to (F) Scanning electron micrographs of the ovule surface of transgenic *GhACT1* RNAi and wild-type plants.

(A) to (C) Ovules of wild-type plants at 1 (A), 2 (B), and 3 (C) DPA. Note the length of fibers increases with time.

(D) to (F) Ovules of transgenic plants at 1 (D), 2 (E), and 3 (F) DPA. Note the length of fibers is much shorter than that in wild-type plants at the same stages.

(G) to (L) Organization of actin filaments in fiber cells of wild-type and transgenic *GhACT1* RNAi cotton.

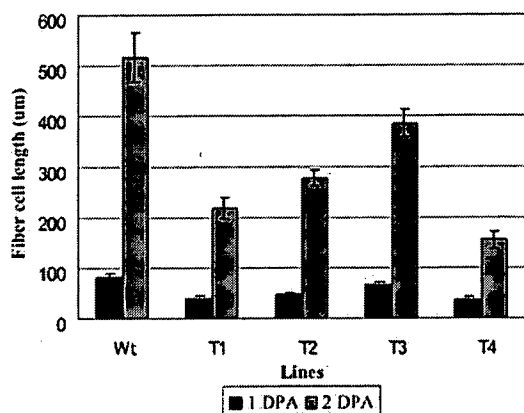
(G) to (I) Fiber cells of wild-type cotton at 1 (G), 2 (H), and 3 (I) DPA. Actin filaments were organized into arrays parallel to the growing axis and extended into the tip of the fiber cells at 1 DPA (G). Actin filaments were arranged into thin arrays and thick cables along the shank in fiber cells at 2 DPA (H) and assumed a more complicated net structure of thick and longitudinally extending cables in >3-DPA fiber cells (I).

(J) to (L) Fiber cells of transgenic cotton at 1 (J), 2 (K), and 5 (L) DPA. Fewer F-actin cables were present.

Bars = 5  $\mu$ m in (G) and (J) and 10  $\mu$ m in (H), (I), (K), and (L).

A unique feature of the actin gene family is the position of introns that are conserved among actin genes in cotton and other plant species (Shah et al., 1983; Baird and Meagher, 1987; Nairn et al., 1988; Stranathan et al., 1989; McElroy et al., 1990; Meagher and Williamson, 1994; Cox et al., 1995; An et al.,

1996). In *Arabidopsis*, actin genes have three small introns at identical locations as in *GhACT* genes except *ACT2*, in which the first intron between codons 20 and 21 is missing (McDowell et al., 1996). In *GhACT1* and *GhACT2*, the first intron is rather large (545 and 565 bp, respectively) compared with the second and third



**Figure 10.** Fiber Length of Transgenic *GhACT1* RNAi and Wild-Type Cotton Seeds at 1 and 2 DPA.

Ovules were sectioned and the length of 30 fiber cells was measured under a microscope for each transgenic line and wild type. Data was processed with Microsoft Excel. Compared with the wild type, fiber cells of transgenic plants are much shorter and are approximately one-half to one-third of wild-type fibers.

introns (91 and 76 bp, respectively) that are more conserved, indicating that the first intron is more divergent than intron 2 and intron 3. Furthermore, unlike the other known plant actin genes studied, *GhACT1* and *GhACT2* share high similarity in the sequences of all three introns, suggesting that both genes may have very close evolutionary relationship.

Nevertheless, the triplet CAG insertion 4 bp upstream the second exon–intron junction in *GhACT8* challenged the conserved intron organization paradigm that the three introns are located at the same positions in all actin genes in the plant kingdom (McDowell et al., 1996). In actin genes examined so far, the second intron is always located at amino acid residue 152. However, in *GhACT8*, the second intron was located at amino acid residue 153 instead of residue 152 because of the insertion. The functional and evolutionary implications of this insertion remain unknown, although *GhACT8* is expressed at high levels in fiber and root.

#### ***GhACT1* Is Preferentially Expressed during Fiber Development**

In this study, we demonstrated that differential expression of the *GhACT* gene occurs in cotton, as did the members of this actin family in several other plant species, such as *Arabidopsis* (McDowell et al., 1996; Meagher et al., 1999b), soybean (McLean et al., 1990), tobacco (Thangavelu et al., 1993), and rice (McElroy et al., 1990). The differential expression data may imply that the specialized functional expressions of actin genes are required for proper development of the respective cell and tissue types and may reflect the divergent evolution of actin gene regulatory elements for expression in plant development.

The data presented here provide evidence for strong expression of some *GhACT* genes in cotton fibers. The high level

of *GhACT* gene expression coincides with the rapid elongation of the fiber cell, suggesting that actin cytoskeleton plays an essential role in fiber elongation. It seems that specialized *GhACT* genes had been evolved to meet the requirement of the actin cytoskeleton for rapid fiber elongation. This is manifested by the fiber-specific expression of *GhACT1*, as well as *GhACT2* and *GhACT5*. Real-time RT-PCR and RNA gel blot analysis showed that the *GhACT1* transcripts accumulated preferentially in developing fibers, whereas only low or undetectable levels of RNAs were found elsewhere. The transcripts of *GhACT1* reach the highest level in young fibers during 8 to 14 DPA, and then there is a gradual and visible decrease of mRNA as the fiber cells developed further. Similarly, genes involved in osmoregulation and cell expansion during fiber development are also expressed at a high level (Orford and Timmis, 1998; Smart et al., 1998; Ruan et al. 2001). Consistently, *GhTua2/3* and *GhTua4* genes increased in abundance from 10 to 20 DPA, whereas *GhTua1* and *GhTua5* transcripts were abundant only through to 14 DPA and dropped significantly at 16 DPA with the onset of secondary wall synthesis (Whittaker and Triplett, 1999). Our previous study indicated that the *GhTUB1* gene was preferentially expressed in the early stage of fiber development (Li et al., 2002). This suggests that strict developmental control on genes, such as *GhACT1*, involved in cell elongation during cotton fiber and ovule development had evolved.

To study the developmental control mechanisms, we isolated the *GhACT1* gene and its promoter. The 0.8-kb 5' upstream sequence was cloned upstream the *GUS* reporter and transferred to cotton plants. *GUS* assay showed that the promoter is very active in developing fibers, whereas no or very little activity is present in leaf, stem, root, petal, and sepal. It should be emphasized that *GhACT1* was not expressed in leaf, stem, root, petal, and sepal trichomes, suggesting that the actin isotype encoded by *GhACT1* may be specific for fiber growth, rather than that of other trichomes. This is consistent with the *GhACT1* expression pattern revealed by real-time RT-PCR and RNA gel blot analysis, indicating that the 0.8-kb *GhACT1* promoter is sufficient to drive its tissue-specific expression and contains all the *cis* regulatory elements for its developmental regulation, as for actin genes in *Arabidopsis* (An et al., 1996; Huang et al., 1996; McDowell et al., 1996; Meagher et al., 1999b; Vitale et al., 2003). Thus, the 0.8-kb *GhACT1* promoter can be useful for isolating transcriptional factors that recognize the promoter sequence and for directing target gene expression in fiber cells. By comparing other fiber-specific promoter sequences such as *E6*, *H6*, and *FbL2A* (John and Crow, 1992; John and Keller, 1995, 1996; Rinehart et al., 1996), we hope to be able to identify fiber-specific *cis* elements and *trans* regulatory factors in the future.

#### ***GhACT1* Plays a Major Role in Fiber Elongation**

Fiber cell development, similar to trichome morphogenesis in leaf and stem (Mathur et al., 1999), requires the actin cytoskeleton for elaborating and maintaining the spatial patterning. During the early stage of fiber elongation, a rapid rate of actin turnover must keep pace with the equally rapid rates of fiber growth. The downregulation of *GhACT1* via RNAi technology in the transgenic fibers greatly reduces actin level that consequently affects



**Figure 11.** Significant Amino Acid Substitutions within Cotton GhACT Proteins.

The model was constructed using the spdbv37sp5 protein structure program (SwissModel first approach mode) from the Web site <http://swissmodel.expasy.org/> of the Swiss Institute of Bioinformatics. Front (A), right side (B), back (C), and left side (D) views, respectively, of the space-filling model for cotton GhACT1. The GhACT1 structure was built based on the known actin three-dimensional structure. Substitutions involving charged or strongly polar amino acid interchanges among the 15 cotton actin isoforms (GhACT1 to GhACT15) are shown in the labeled amino acid residues.

actin cytoskeleton organization, and as a result, fiber elongation is inhibited (Figure 9). This demonstrated that *GhACT1* plays a major role in fiber elongation, although we could not completely rule out the contribution of *GhACT* genes (such as *GhACT2* and *GhACT5*). The growth of cotton fiber cells is different from that of most other plant cells because of its rapid and synchronous tip elongation. It has been reported that F-actin plays an important role in pollen tube growth (Mascarenhas, 1993; Chen et al., 2002), in trichome morphogenesis (Mathur et al., 1999), in root hair tip growth (Miller et al., 1999), and in cell elongation of other cell types (Baluska et al., 2000; Waller et al., 2002; Yamamoto and Kiss, 2002). In the tip-growing pollen tube, F-actin arrays are very dynamic, changing from large spherical bodies to F-actin bundles oriented predominantly parallel to the growth axis (Tiwari and Polito, 1988). Similar F-actin arrays were also found

for root hair growth (Miller et al., 1999) as well as root tip growth (Blancaflor and Hasenstein, 1997). However, *Arabidopsis act7* mutants showed remarkably reduced F-actin in the cells of the root elongation zone. As a result, mutants displayed a series of abnormal phenotypes, such as delayed and less efficient germination, increased root twisting and waving, and retarded and slowed root growth (Gilliland et al., 2003). The *act2-1* insertion fully disrupted *ACT2* gene expression and significantly decreased the level of total actin protein, resulting in much shorter root hairs (Gilliland et al., 2002; Ringli et al., 2002). Immunocytochemical analysis revealed only several thin actin bundles in the short mutant root hairs, and in the very apex of these stunted root hairs, the actin bundles were often looping through the tip or showing dense but diffuse fluorescence labeling (Gilliland et al., 2002). It was believed that actin isoforms of *Arabidopsis* have



evolved distinct reproductive and vegetative functions and have showed functional nonequivalency among each other. Mis-expression of the pollen-specific reproductive ACT1 isovariant in vegetative tissues altered actin polymerization and F-actin organization and thereby dramatically affects plant development and morphogenesis (Kandasamy et al., 2002). In cotton fiber, we found that the F-actin was organized into short and thin arrays in tip orientation at the early stage of fiber development, and with further development, F-actin cytoskeleton displayed an increasingly complicated net-like structure consisting predominantly of thick and longitudinally extending cables in fiber cells. On the other hand, when GhACT1 isovariant level was reduced significantly in the transgenic fiber cells, F-actin bundles were reduced with only a few filaments (Figure 9), similar to *act7* and *act2* mutants. The reduced and defective actin cytoskeleton was unable to meet rapid fiber elongation. This suggested that F-actin arrays maintained by a significant amount of actins (mostly GhACT1) is critical for fiber cell elongation, like in root hair and trichome cell types.

F-actin might transport vesicles toward the cell periphery, especially near the polar region during tip growth. When the movement of F-actins was blocked, the vesicles could not be released in the cell periphery (polar region), resulting in the inhibition of the coleoptile cell elongation (Waller and Nick, 1997; Waller et al., 2002). In root hair development of *Vicia sativa*, the elongating net-axial fine bundles of actin filaments (FB-actin) function in polar growth by targeting and releasing Golgi vesicles to the vesicle-rich region of the hair cells. When the elongation of FB-actin was blocked by cytochalasin D, the tip growth of root hair cells was stopped (Miller et al., 1999). In our study, because of suppression of *GhACT1* expression, the reduction of F-actin level in transgenic fibers might have a similar effect on fiber cells. With the reduction of actin filaments, the number of organelles (such as Golgi body and endoplasmic reticulum) traveling along the filaments may decrease significantly in the *GhACT1* RNAi transgenic fiber cells. The significant reduction in vesicles may account for the slow elongation of fiber cells in the transgenic plants.

In conclusion, our results provide direct evidence that *GhACT1*, perhaps as well as other *GhACT* genes (such as *GhACT2* and *GhACT5*), is involved in fiber elongation, not fiber initiation. The characterization and expression studies give us novel insights into the role of *GhACT1* in cotton fiber development. Furthermore, the *GhACT1* promoter provides a useful tool to identify transcription regulators confirming its fiber-specific expression and to direct potential target genes for fiber quality improvement.

## METHODS

### Plant Materials

Cotton (*Gossypium hirsutum* cv Coker312) seeds were surface-sterilized with 70% ethanol for 30 to 60 s and 10% H<sub>2</sub>O<sub>2</sub> for 30 to 60 min, followed by washing with sterile water. The sterilized seeds were germinated on half-strength MS medium under a 12-h-light/12-h-dark cycle at 28°C. Cotyledons and hypocotyls were cut from sterile seedlings as explants for transformation as described before (Li et al., 2002). Tissues for DNA and

RNA extraction were derived from cotton plants (*G. hirsutum* cv DP5415 and Xuzhou142) grown in a greenhouse.

### Construction of Cotton cDNA Libraries

Total RNA was extracted from young fibers, ovules, anthers, petals, leaves, cotyledons, and roots as described previously (Li et al., 2002). Poly(A)<sup>+</sup> mRNA was purified from total RNA using an mRNA purification kit (Qiagen, Düsseldorf, Germany). cDNA was synthesized and cloned into the *EcoRI*-*XhoI* sites of the ZAP Express vector and packaged using a ZAP-cDNA Gigapack Gold III cloning kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

### Isolation of *GhACT* cDNAs and RNA Gel Blot Analysis

More than 300 cDNA clones were randomly selected from the cotton fiber cDNA library for sequencing. Sequence analysis identified one actin clone, *GhACT1*. The 380-bp fragment of the 3'-UTR of *GhACT1* was obtained by PCR amplification using primers *GhACT1*-3'L (5'-AGTTTGTGTAATTGCTTTTGATGGT-3') immediately downstream the stop codon and *GhACT1*-3'R (5'-AAATCTCGTACAATAATAGCTATT-3') and used as a gene-specific probe for RNA gel blot analysis as described previously (Li et al., 2002). Then, a 600-bp fragment representing *GhACT1* exon 3 was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP and used as a probe to screen a cotton cDNA library according to standard procedures (Sambrook et al., 1989). cDNA (5 × 10<sup>6</sup>) clones were screened, and 300 clones were identified. Among them, 60 full-length clones were sequenced and analyzed. In total, 15 unique cDNA clones were obtained.

### Sequence and Phylogenetic Analysis

Nucleotide and amino acid sequences were analyzed using DNASTAR (DNASTAR, Madison, WI). For phylogenetic analysis, 15 *GhACT* peptide sequences and one putative cotton actin sequence (AF059484) were aligned with the ClustalW program (<http://www.ebi.ac.uk>), then maximum parsimony analysis was performed with the PAUP 4.0 program (Swofford, 1998) using yeast actin ScACT1 as an outgroup. The heuristic search methods were applied and the best parsimonious trees were retained in each search.

### RT-PCR Analysis

The expression of the *GhACT* genes in cotton tissues was analyzed by real-time quantitative RT-PCR using the fluorescent intercalating dye SYBR-Green in a LightCycler detection system (Roche, Indianapolis, IN). A cotton polyubiquitin gene (*GhUBI*) was used as a standard control in the RT-PCR reactions. A two-step RT-PCR procedure was performed in all experiments. First, total RNA samples (2 µg per reaction) from leaves, stems, cotyledons, roots, anthers, petals, and fibers were reversely transcribed into cDNAs by AMV reverse transcriptase according to the manufacturer's instructions (Roche). Then, the cDNAs were used as templates in real-time PCR reactions with gene-specific primers (Table 1). The real-time PCR reaction was performed using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. The amplification of the target genes was monitored every cycle by SYBR-Green fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure for the starting copy numbers of the target gene. Relative quantitation of the target *GhACT* expression level was performed using the comparative Ct method (Roche LightCycler system). The relative value for expression level of each *GhACT* gene was calculated by the equation  $Y = 10^{\Delta Ct/3} \times 100\%$  ( $\Delta Ct$  is the differences of Ct between the control *GhUBI* products and the target

*GhACT* products; i.e.,  $\Delta Ct = Ct_{GhUBI} - Ct_{GhACT}$ ). To achieve optimal amplification, PCR conditions for every primer combination were optimized for annealing temperature and  $Mg^{2+}$  concentration as recommended by the Roche LightCycler system instructions. PCR products were confirmed on an agarose gel. The efficiency of each primer pair was detected using *GhACT* cDNAs as standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair.

#### DNA Gel Blot Analysis

Genomic DNA was isolated from young cotton (*G. hirsutum* cv DP5415 and Xuzhou142) leaves using a modified method described earlier (Li et al., 2002). Genomic DNA was digested with restriction enzymes and separated on 0.7% agarose gels and transferred onto Hybond N<sup>+</sup> nylon membranes (Amersham Biosciences, Buckinghamshire, UK) by capillary blotting. DNA gel blot hybridization was performed at 68°C overnight using ExpressHyb solution (Clontech, Palo Alto, CA) with <sup>32</sup>P-labeled gene-specific DNA probes prepared by the Prime-a-Gene labeling system (Promega, Madison, WI), followed by washing at 68°C in 0.1× SSC and 0.5% SDS for 30 to 60 min. The <sup>32</sup>P-labeled membranes were exposed to x-ray film at −80°C for 1 to 3 d.

#### Isolation of *GhACT* Genes by Screening Cotton Genomic Libraries

Cotton genomic libraries were constructed as described earlier (Li et al., 2002). Approximately  $2 \times 10^6$  clones were screened with a [ $\alpha$ -<sup>32</sup>P]dCTP-labeled *GhACT1* (0.6 kb of exon 3) probe generated by the Prime-a-Gene labeling system (Promega). The membranes (Hybond N<sup>+</sup>; Amersham Biosciences) were hybridized overnight in ExpressHyb solution (Clontech) at 68°C, followed by washing with 0.1× SSC and 0.5% SDS. Autoradiography was performed with x-ray film (Kodak, Rochester, NY), and positive clones were purified and sequenced with the ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

#### Construction of the *GhACT1::GUS* Chimeric Gene and *GhACT1* RNAi

Primers at −816 to −793 bp with an introduced *Sa*I site and from −1 to −27 bp before ATG with an introduced *Xba*I site were used to amplify the *GhACT1* promoter. A 0.8-kb PCR fragment was obtained using pfu DNA polymerase (Stratagene, La Jolla, CA) and digested with *Sa*I and *Xba*I, then subcloned into the *Sa*I/*Xba*I sites of the pBI101 vector (Clontech) to generate a chimeric *GhACT1::GUS* gene (named pBI-ACT1-p) (Figure 4B).

To construct *GhACT1* RNAi vector, the first intron (0.2 kb) of the *GhTUB1* gene (Li et al., 2002) was amplified by PCR with two introduced sites, *Xba*I and *Spe*I, using the primer pair TUBint-L, 5'-GGGTCTAGAGACGTAGTTAGAAAGGAAGCCGA-3', and TUBint-R, 5'-GGGACTAGTACGTTCCCATTCGGGAACCCGTT-3', and inserted into a pBluescript II SK<sup>+</sup> vector at the sites *Xba*I and *Spe*I to obtain an intron-containing intermediate construct (pSK-TUBint). The *GhACT1* 3'-terminal sequence (150 bp fragment at 229 to 378 bp downstream the stop codon) was cloned into the 5' arm with the introduced sites *Bam*HI/*Spe*I and the 3' arm with the introduced sites *Xba*I/*Sac*I of the intron in pSK-TUBint vector for the sequences encoding the inverted repeat RNA. The constructed RNAi of the *GhACT1* gene was subcloned into the *GhACT1::GUS* construct at *Bam*HI/*Sac*I sites to replace the *GUS* gene (named pBI-TUBint-ACT1i) (Figure 4C).

#### Cotton Transformation

Cotyledon and hypocotyl explants from *G. hirsutum* cv Coker 312 were transformed using *Agrobacterium tumefaciens*-mediated transformation as described previously (Li et al., 2002). Homozygosity of transgenic

plants was determined by segregation ratio of the kanamycin selection marker and further confirmed by DNA gel blot analysis.

#### Histochemical Assay of *GUS* Gene Expression

Histochemical assays for *GUS* activity in transgenic cotton plants were conducted according to Jefferson et al. (1987), with slight modification. Fresh plant tissues were incubated in 5-bromo-4-chloro-3-indolylglucuronide solution at 37°C for 4 to 8 h and then cleared and fixed by rinsing with 100 and 70% ethanol successively. For sectioning, 1 to 3 DPA ovules stained in 5-bromo-4-chloro-3-indolylglucuronide solution were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, overnight at room temperature, then dehydrated through conventional ethanol series, and finally embedded in Histo-resin (Leica, Wetzlar, Germany) according to the manufacturer's instructions. The samples were cut into 5- to 7- $\mu$ m-thick sections using a Leica microtome. The sections were examined and photographed under a Leica DMR microscope equipped with dark-field optics.

#### Protein Gel Blot Analysis

Soluble proteins were extracted from 8 to 10 DPA wild-type and *GhACT1* RNAi transgenic fibers in extraction buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 mM  $CaCl_2$ ,  $\alpha$ -mercaptoethanol, 0.5% Nonidet P-40, 1  $\mu$ g/mL aprotinin, 1  $\mu$ g/mL leupeptin, and 0.6  $\mu$ L/mL PMSF. Protein concentration was determined by the Bradford method. Equal amounts of proteins were separated by electrophoresis in a 12% SDS-PAGE gel and transferred onto a nylon membrane by electric transfer (Trans-Blot system; Bio-Rad, Hercules, CA) using semidry transfer buffer. The membrane was blocked with 5% nonfat milk in PBS buffer containing 0.05% Tween-20 at room temperature for at least 1 h and then incubated with affinity-purified goat polyclonal anti-actin IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After washing in PBS buffer for 30 min, the membrane was incubated in PBS containing horseradish peroxidase-conjugated rabbit anti-goat IgG (Pierce, Rockford, IL) for 1 h. After washing in PBS buffer, the membrane was incubated in SuperSignal West Substrate (Pierce) working solution for 5 min and then exposed to x-ray film.

#### Scanning Electron Microscopy

For examining fiber initiation and elongation, fresh ovules were dissected out and placed on double-sided sticky tape on an aluminum specimen holder and frozen immediately in liquid nitrogen. The frozen sample was viewed with a JSM-5310LV scanning electron microscope (JEOL, Tokyo, Japan).

#### Observation of F-Actin Structures in Fiber Cells

Ovules dissected out from fresh bolls at 1 to 4 DPA, with or without maleimidoenzoyl-*N*-hydroxysuccinimide ester pretreatment, were fixed in a solution of 2% paraformaldehyde in KMCP buffer (70 mM KCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , and 100 mM Pipes, pH 6.5) for 1 h. After rinsing in KMCP buffer, the ovules were sectioned into slices of  $\sim 1$  mm thickness. Thin sections were transferred to slides and treated with 1% cellulase (Sigma-Aldrich, St. Louis, MO), 0.5% hemicellulase (Sigma-Aldrich), 0.5% pectinase (Sigma-Aldrich), and 0.1% BSA in KMCP buffer for 10 min, followed by washing with KMCP buffer. Finally, sections were incubated in a solution of 5  $\mu$ g/mL Phalloidin-TRITC (Sigma-Aldrich) in KMCP buffer with 0.1% Triton X-100 at room temperature. Excess phalloidin was removed by rinsing with the same buffer. Stained ovule sections were immediately examined with an LSM510 confocal microscope (Zeiss, Jena, Germany).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY305723 to AY305737.

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# Exhibit 18

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## Molecular analysis of the stylar-expressed *Solanum chacoense* small asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*

M. O'Brien<sup>1</sup>, C. Kapfer<sup>1</sup>, G. Major<sup>1</sup>, M. Laurin<sup>1</sup>, C. Bertrand<sup>1</sup>, K. Kondo<sup>2</sup>, Y. Kowiyama<sup>2</sup> and D. P. Matton<sup>1,\*</sup>

<sup>1</sup>Département de Sciences Biologiques, Institut de recherche en biologie végétale, Université de Montréal, 4101 Sherbrooke est, Montréal, Québec, Canada H1X 2B2, and

<sup>2</sup>Faculty of Bioresources, Mie University, Tsu 514-8507, Japan

Received 12 July 2002; revised 20 August 2002; accepted 16 September 2002.

\*For correspondence (fax +1514 8729406; e-mail mattond@irbv.umontreal.ca).

### Summary

Gametophytic self-incompatibility (GSI) systems involving the expression of stylar ribonucleases have been described and extensively studied in many plant families including the Solanaceae, Rosaceae and Scrophulariaceae. Pollen recognition and rejection is governed in the style by specific ribonucleases called S-RNases, but in many self-incompatibility (SI) systems, modifier loci that can modulate the SI response have been described at the genetic level. Here, we present at the molecular level, the isolation and characterization of two *Solanum chacoense* homologues of the *Nicotiana* HT modifier that had been previously shown to be necessary for the SI reaction to occur in *N. alata* (McClure *et al.*, 1999). HT homologues from other solanaceous species have also been isolated and a phylogenetic analysis reveals that the HT genes fall into two groups. In *S. chacoense*, these small proteins named ScHT-A and ScHT-B are expressed in the style and are developmentally regulated during anthesis identically to the S-RNases as well as following compatible and incompatible pollination. To elucidate the precise role of each HT isoform, antisense ScHT-A and RNAi ScHT-B lines were generated. Conversion from SI to self-compatibility (SC) was only observed in RNAi ScHT-B lines with reduced levels of ScHT-B mRNA. These results confirm the role of the HT modifier in solanaceous SI and indicate that only the HT-B isoform is directly involved in SI.

**Keywords:** self-incompatibility, S-RNase, Solanaceae, HT-modifier gene, RNA interference.

### Introduction

Self-incompatibility (SI) constitutes an important mechanism for preventing inbreeding through specific pollen recognition and rejection. In the most widespread type of gametophytic self-incompatibility (GSI), the haploid pollen is rejected when the S-allele it expresses, matches either of the two S-alleles expressed in the sporophytic tissue of the pistil. For *Solanaceae*, the GSI phenotype is specified by a highly multiallelic S-locus (de Nettancourt, 1977, 1997) whose only known product is a secreted ribonuclease (McClure *et al.*, 1989) expressed in the transmitting tissue of the style (Anderson *et al.*, 1986; Matton *et al.*, 1998) and called an S-RNase. Gain-of-function experiments in SI plants have shown that expression of an S-RNase transgene is sufficient to alter the SI phenotype of the pistil but

not that of pollen (Lee *et al.*, 1994; Matton *et al.*, 1997; Murfett *et al.*, 1994). Furthermore, transgenic plants made to express high levels of S-RNase in pollen did not acquire the new phenotype (Dodds *et al.*, 1999), indicating that the pollen S gene (unknown to date) is clearly distinct from the S-RNase (Kao and McCubbin, 1996). In order to determine if expression of an active S-RNase is the sole determinant of SI in styles, transformation of closely related self-compatible (SC) species with S-RNases were attempted. Transformation of SC *Nicotiana tabacum* or *N. plumbaginifolia* with an S-allele from the SI species *N. alata* did not result in the acquisition of the SI phenotype (Murfett *et al.*, 1996), nor did the introgression of a chromosome fragment bearing the S-locus from the SI *Lycopersicon hirsutum* in SC

*L. esculentum* (Bernatzky *et al.*, 1995), or the expression of an S-RNase from the SI *L. peruvianum* in the SC *L. esculentum* (Kondo *et al.*, 2002b). Conversely, when an S-allele from the SC *Petunia hybrida* was introduced in SI *P. inflata*, it became functional in rejecting its corresponding self-pollen, indicating that factors expressed in the *P. inflata* SI genetic background were needed for the SI reaction to occur (Ai *et al.*, 1991). These results strongly suggest that other factors are necessary for the SI reaction to occur. Some of these factors that affect the SI response have been described in numerous SI systems and often been named S-locus inhibitors or modifiers (de Nettancourt, 1977). In *S. chacoense*, an S-locus inhibitor (Sli) has been mapped to the distal end of chromosome 12, but has not been cloned yet (Hosaka and Hanneman, 1998a,b). To date, the only modifier functionally characterized at the molecular level is the HT gene, a stylar-expressed small asparagine-rich protein in *N. alata* (McClure *et al.*, 1999). The NaHT (*Nicotiana alata* HT) cDNA was isolated from a differential screen for SI stylar-specific transcripts, and antisense *Nicotiana* HT plants became SC, although they still expressed normal levels of stylar S-RNases. Recent correlative evidences from mRNA expression studies in *Lycopersicon* species also suggest the involvement of the HT modifier in SI (Kondo *et al.*, 2002a,b). Here, we describe the characterization of HT homologues that are co-ordinately expressed with the S-RNases during pistil development in the SI species *S. chacoense*, and show that only the HT-B isoform is involved in SI.

## Results

### Isolation of the *Solanum* HT homologues and sequence comparison

The ScHT-A<sub>1</sub>, ScHT-A<sub>2</sub> and S<sub>14</sub>-RNase cDNAs were isolated from a pollinated pistil cDNA library (see Experimental procedures section). The ScHT-A<sub>1</sub> cDNA codes for a small protein of 99 amino acid residues with a highly predicted N-terminal signal peptide as determined from the SignalP algorithm (Nielsen *et al.*, 1997). The predicted cleavage site for ScHT-A<sub>1</sub> is before Arg-25, producing a mature polypeptide of 75 amino acids (8 kDa). The ScHT-A<sub>2</sub> cDNA is incomplete in the 5' region, but would comprise all of the mature protein (77 residues, 8.3 kDa) as predicted from the ScHT-A<sub>1</sub>-deduced cleavage site. Both ScHT-A<sub>1</sub> and ScHT-A<sub>2</sub> predicted mature proteins are acidic with pIs of 3.98 and 4.11, respectively. Amino acid sequence comparison of the predicted mature polypeptides indicate that ScHT-A<sub>1</sub> and ScHT-A<sub>2</sub> are 96% identical (93% nucleotide sequence identity) and most probably correspond to allelic variants of the same gene (see linkage analysis of the ScHT-A isoforms below). The ScHT-B<sub>1</sub> isoform was obtained by PCR ampli-

fication with an upstream primer located in the signal peptide region and a downstream primer located 3' of the predicted stop codon from the *N. alata* HT and *S. chacoense* HT-A<sub>1</sub> isoforms. The ScHT-B<sub>1</sub> mature protein comprises 79 amino acids (MW, 8.7 kDa) with an acidic pI of 4.67, and is approximately 51% identical (57% similar) at the amino acid level to the ScHT-A isoforms. No N-glycosylation sites are found on either polypeptides, but six cysteine residues that could be involved in disulfide bonding are conserved between all HT homologues, except from the *S. pinnatisectum* B<sub>1</sub> isoform that lacks one cysteine, and are found flanking a striking C-terminal region containing 16–20 Asp (D) or Asn (N) residues. In the mature ScHT proteins, asparagine and aspartic acid residues account for roughly 30% of the total amino acids. A sequence alignment of the deduced amino acid sequences corresponding to the mature protein region of the *S. chacoense* HT isoforms as well as HT homologues from other SI solanaceous plants, including *L. peruvianum*, *N. alata*, *S. pinnatisectum*, *S. bulbocastanum* and from the SI species *S. tuberosum*, is shown in Figure 1(a). All *Solanum* and *Lycopersicon* sequences were obtained by PCR amplification with the same primer pairs as described for the amplification of ScHT-B<sub>1</sub>. Although all the HT sequences share some specific structural features, e.g. a C-terminal Asn/Asp-rich region flanked by conserved cysteine residues, they can be easily classified in two groups when the amino-terminal half of the protein is considered. Based on the CLUSTALX alignment, a phylogenetic analysis was performed to determine if this preliminary classification would hold true. Figure 1(b) shows that all the B isoforms fell into a highly supported cluster, while more sequence data would be needed to determine if the A-type sequences form one or more group. Interspecific amino acid sequence identities between the predicted mature polypeptides ranges from 76 to 86% in the A-isoform group, and 36–92% in the B-isoform group. The ScHT-A<sub>1</sub> and ScHT-A<sub>2</sub> (94%), SbHT-B<sub>1</sub> and SbHT-B<sub>2</sub> (98%) and SpHT-B<sub>1</sub> and SpHT-B<sub>2</sub> (97%) are most probably alleles of the same genes in their respective species. When the only non-*Solanum* sequence is removed (NaHT-B), the B-isoform group sequence identity is in the range of 77–92%. One surprising feature is the very high conservation of the predicted signal peptides between species, as determined from the available complete HT cDNA sequences (ScHT-A<sub>1</sub>, NaHT-B, LpHT-A<sub>1</sub> and LpHT-B<sub>1</sub>), ranging from 66 to 100% identity (82–100% similarity), when compared to the mature protein sequences (data not shown). This intriguing situation is also observed with the sporophytic SI (SSI) pollen S gene where the signal peptides are also far more similar to each other (mean of 77% identity and 89% similarity) than the mature protein sequences (29% identity and 38% similarity on average) when the sequences of five different SSI pollen S genes are compared (Schopfer *et al.*, 1999; Takayama *et al.*, 2000).

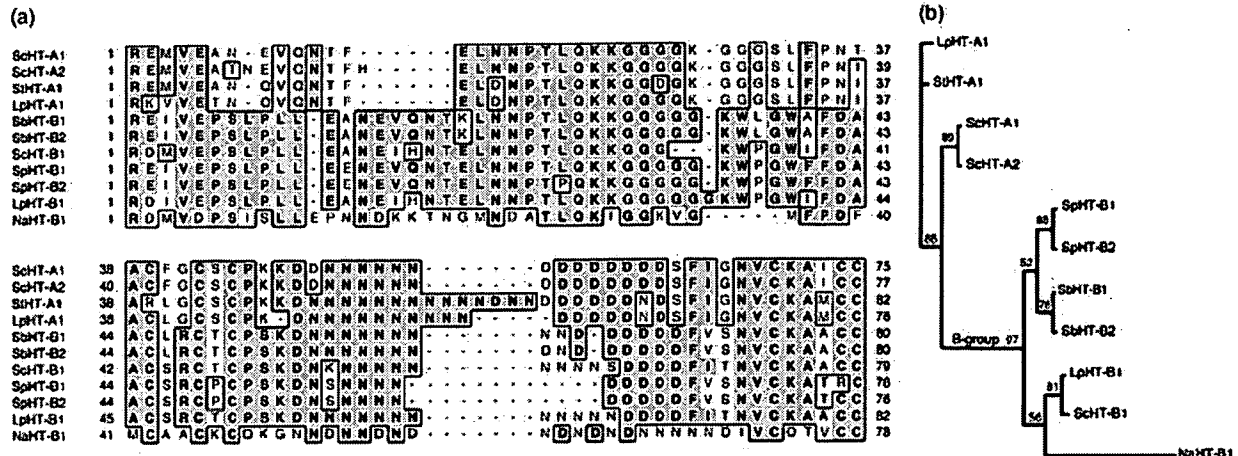


Figure 1. Sequence alignment (a) and phylogenetic analysis (b) of the deduced mature protein sequences of *ScHT-A1*, *ScHT-A2* and *ScHT-B1* with related sequences from other solanaceous species.

(a) CLUSTALX alignment was used to produce a phylogenetic analysis of related HT sequences in six solanaceous species.

(b) A jackknife analysis using Paup 4.08b was used to produce the phylogram which is shown.

#### Tissue-specific and developmental regulation of the *ScHT* modifiers

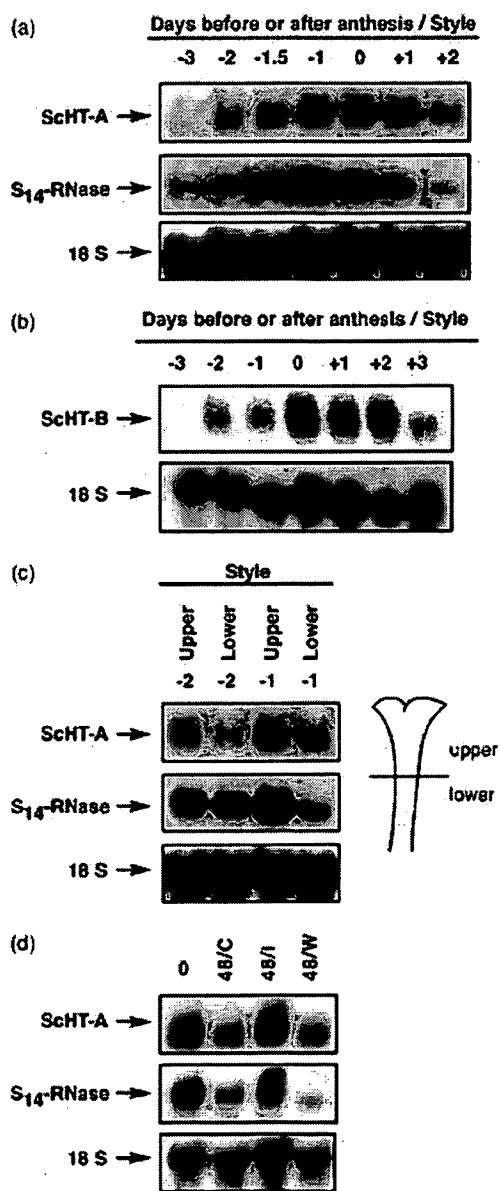
Tissue-specific expression of *ScHT-A* and *ScHT-B* isoforms was determined using RNA extracted from different tissues of *S. chacoense*. Since the *ScHT-A1* and *ScHT-A2* cDNAs are 93% identical at the DNA level, the RNA-gel blot analyses most probably reflect the expression of both genes, although the probe used at all time was *ScHT-A1*. Overall DNA sequence identity between the *ScHT-A* and *ScHT-B* isoforms is around 73%, and long stretches of identity might also produce cross-hybridization. In order to avoid this, an oligonucleotide specific to the B isoform and corresponding to the N-terminal sequence, PSLPLLEA, was synthesized. Both *ScHT-A* and *ScHT-B* isoforms are almost exclusively expressed in styles with very weak expression detected in ovary upon prolonged exposures (data not shown). No *ScHT-A* or *B* mRNAs could be detected in leaf, stem, root, petal, anther, pollen or pollen tube tissues (data not shown). This expression pattern is identical to the one observed for the *S-RNase*s (Matton *et al.*, 1998). Since the *S-RNase* genes are themselves developmentally regulated during anthesis (Anderson *et al.*, 1986; Cornish *et al.*, 1987), we determined the RNA expression pattern of *ScHT-A* and *ScHT-B*, and compared with the one obtained from *S14-RNase* (Figure 2a,b). Both *ScHT* isoforms and the *S14-RNase* are identically regulated during pistil development and reach a maximum level of expression around anthesis day (Figure 2a,b). Figure 2(a,b) also shows that, in unpollinated flowers, *ScHT-A*, *ScHT-B* and *S14-RNase* mRNA levels decline from around 2 days after anthesis, coinciding with a reduced fertilization receptivity.

In *S-RNase*-mediated GSI, rejection of the pollen tubes mostly occurs in the top half of the style. To determine if

there could be a correlation with pollen tube arrest and the expression levels of genes involved in SI, mRNA levels of *ScHT-A* and *S14-RNase* were measured in the upper and lower halves of styles around peak expression time (Figure 2c). Both genes were more strongly expressed in the upper half of the style, consistent with the site of most pollen tube arrest as determined by aniline blue staining in *S. chacoense* styles (Matton *et al.*, 1999).

#### Effect of compatible and incompatible pollination on *ScHT* and *S-RNase* gene expression

In many species, pollination is known to induce deterioration and death of the secretory cells in the stigmatic region and in the transmitting tissue of the style (Cheung, 1996). We have previously shown that some genes that respond to pollination, also respond to wounding stress and wound hormone treatments, mainly jasmonates (Lantin *et al.*, 1999a,b). Wounding, as well as wound hormone treatment (JA, ABA, MeJA) and elicitors of defense responses (salicylic acid, arachidonic acid), had no effect on either *ScHT-A* or *S14-RNase* mRNA levels (data not shown, except for wounding in Figure 2d). Expression of these genes thus seemed to be exclusively controlled by developmental cues during pistil maturation, except for a differential response toward the type of pollination. *ScHT-A* and *S-RNase*s responded differentially to a compatible or an incompatible pollination. In Figure 2(d), flowers were pollinated with either compatible or incompatible pollen and tissues were harvested 48 h later. For the wounding treatment, the upper part of the style including the stigma was slightly crushed with tweezers and tissues were also harvested 48 h later. Following a compatible pollination, or wounding, both *ScHT-A* and *S14-RNase* mRNA levels declined similar to



**Figure 2.** RNA expression analysis of *ScHT* transcript levels in styles. (a) Developmental expression pattern of *ScHT-A* and *S<sub>14</sub>-RNase* mRNA levels in unpollinated pistil tissues. *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels were determined by RNA-gel blot analysis of unpollinated pistil tissues, 3 days before anthesis (-3) to 2 days (+2) after anthesis. Ten micrograms of total style RNA from each developmental stage was probed with the *ScHT-A*<sub>1</sub> cDNA insert, stripped and re-probed with the *S<sub>14</sub>-RNase* cDNA insert. (b) Developmental expression pattern of *ScHT-B* mRNA levels in unpollinated pistil tissues. Same conditions as in (a), except that an identical RNA-gel blot was probed with the *ScHT-B*<sub>1</sub> specific oligonucleotide. (c) Differential expression of *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels in upper and lower part of the style. *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels were determined by RNA-gel blot analysis in upper and lower halves of styles collected 2 (-2) and 1 day (-1) before anthesis. Conditions same as in (a). (d) Effect of compatible and incompatible pollination on *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels. *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels were determined by RNA-gel blot analysis in unpollinated styles at anthesis day (0), in styles collected 48 h after a fully compatible (S<sub>11</sub>S<sub>12</sub> × S<sub>13</sub>S<sub>14</sub>) pollination

the developmentally regulated decrease observed in unpollinated flowers (compare Figure 2a,d). An incompatible pollination had the opposite effect. The *ScHT-A* and *S<sub>14</sub>-RNase* mRNA levels stayed as high as found on anthesis day, indicating that the developmentally programmed decrease in *S-RNase* and *ScHT* mRNA levels could be reversed, at least transiently, following an incompatible pollination.

#### Polymorphism of the HT modifiers and linkage to the *S*-locus

Using the *ScHT-A*<sub>1</sub> cDNA insert as a probe, an F<sub>1</sub> population from a parental cross (S<sub>11</sub>S<sub>12</sub> × S<sub>13</sub>S<sub>14</sub>) was tested for polymorphism and linkage to the *S*-locus. A fraction of the F<sub>1</sub> progeny tested is shown in Figure 3. The *S-RNase* genotype of the progeny had been determined previously (Rivard *et al.*, 1994) and was confirmed by PCR analyses with allele-specific primers (data not shown). The *ScHT-A* gene is highly polymorphic as four different RFLPs could be detected in these plants. Although four different *S*-alleles also segregated in this population, the *ScHT-A* alleles were completely unlinked to the *S*-locus, as any combination of *ScHT-A* alleles could be found with all four *S-RNases* in this population. The same population was re-probed with the *ScHT-B* cDNA. Two new RFLPs specific to the B form were observed (data not shown). Although cross-hybridization does occur between the *ScHT-A* and *ScHT-B* cDNA probes (data not shown), no single RFLP could be linked with the *S-RNase* gene.

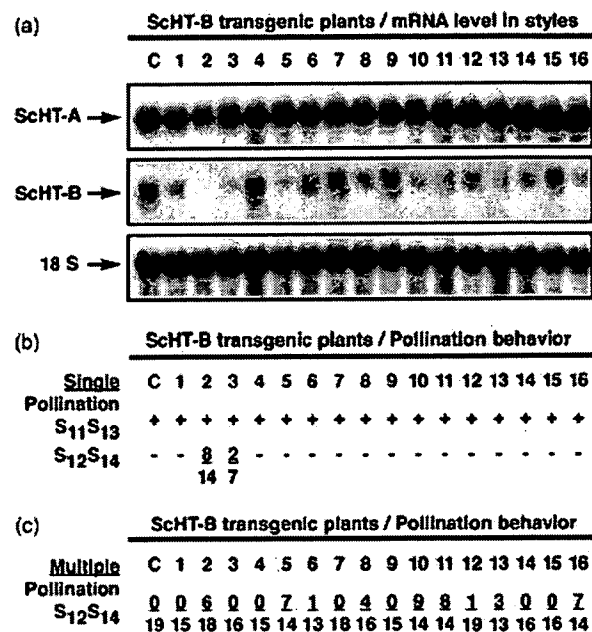
#### Two-hybrid analysis of *ScHT* and *S-RNase* protein interaction

A few putative roles have been proposed for the *N. alata* HT protein (McClure *et al.*, 1999). Recently it was shown that *S-RNases*, in both compatible or incompatible interactions, are taken up by pollen tubes, but the entry mechanism is still unknown (Luu *et al.*, 2000). One possibility is that other stylar factors involved in SI, such as the HT protein, could accompany or interact directly with the *S-RNases* as they are being transported into the growing pollen tubes. Since HT proteins from either *S. chacoense* or *N. alata* are fairly acidic proteins with pI around 4, and since *S-RNases* are basic proteins (*S<sub>14</sub>-RNase* mature protein, predicted pI is 9.12), *ScHT* proteins could interact directly with *S-RNases*, albeit not in a sequence-specific manner, as determined by the linkage analysis (Figure 3). Another possibility would be that the HT proteins could interact with the pollen tubes and

**Figure 2. continued**  
(48/C), in styles collected 48 h after a fully incompatible (S<sub>13</sub>S<sub>14</sub> × S<sub>13</sub>S<sub>14</sub>) pollination (48/I) and in styles collected 48 h after wounding (48/W). Conditions same as in (a). To ascertain equal loading conditions, all RNA-gel blots were stripped and re-probed with an 18S ribosomal cDNA probe from *S. chacoense*.







**Figure 5.** RNA expression analysis of *ScHT* transcript levels in styles of *ScHT-B* RNAi transgenic plants and genetic cross results.

(a) *ScHT-A* and *ScHT-B* transcript levels were determined by RNA-gel blot analysis of unpollinated styles at anthesis day from 16 RNAi *ScHT-B*<sub>1</sub> transgenic plants. Control plant (C) is the untransformed host (genotype S<sub>12</sub>S<sub>14</sub>). Ten micrograms of total style RNA from each plant was probed with the *ScHT-A*<sub>1</sub> cDNA insert and the *ScHT-B*<sub>1</sub> specific oligonucleotide. Equal loading conditions were verified with an 18S ribosomal cDNA probe from *S. chacoense*.

(b) Genetic cross results with pollen from SI or SC plants. All crosses were done on at least 10 flowers per plant on anthesis day, with pollen from a fully compatible plant (genotype S<sub>11</sub>S<sub>13</sub>) or a fully incompatible plant (genotype S<sub>12</sub>S<sub>14</sub>). The plus (+) sign indicates a fully compatible pollination: crosses were successful and seed set occurred in more than 90% of the pollinated flowers. The minus (-) sign indicates a fully incompatible pollination: no crosses were successful and no seed set occurred. For intermediate phenotypes, the number of successful pollination leading to seed set per flower pollinated is indicated.

(c) Genetic cross results with pollen from a SI plant after repeated pollination (multiple pollination). Same as in (b) except that the flowers were repeatedly pollinated with pollen from a fully SI parent (genotype S<sub>12</sub>S<sub>14</sub>). Pollination was done on anthesis day, and then after 24, 48 and 72 h.

transcripts (*ScHT-A*AS plant #9, as determined by densitometric scans), and since correlative evidences showing weak or complete loss of expression of *HT-B* homologues, but not of *HT-A* homologues in some SC *Lycopersicon* species, have been recently obtained (Kondo et al., 2002a,b), we also decided to target the *ScHT-B* gene through an RNA interference (RNAi) strategy. The *ScHT-B*<sub>1</sub> cDNA was inserted first in the sense orientation downstream of the CaMV 35S promoter, followed by a 327-bp spacer, and by the *ScHT-B*<sub>1</sub> cDNA again, but in the antisense orientation. This RNAi construct was then inserted in the *A. tumefaciens* LBA4404 strain and used to transform *S. chacoense* plants of the S<sub>12</sub>S<sub>14</sub> genotype. Sixteen primary transformants were initially selected. All *ScHT-B* RNAi lines were cross-pollinated with pollen from fully compatible

(S<sub>11</sub>S<sub>13</sub>) or fully incompatible (S<sub>12</sub>S<sub>14</sub>) genotypes. Two plants (#2 and #3) sired seeds upon self-pollination (pollen from genotype S<sub>12</sub>S<sub>14</sub>), and could be scored as partially or semi-compatible (Figure 5b). *ScHT-A* and *ScHT-B* mRNA levels were then determined in mature flowers at anthesis. Figure 5a shows an RNA-gel blot of all the transgenic plants probed with either the *ScHT-A*<sub>1</sub> complete cDNA or the *ScHT-B*<sub>1</sub>-specific oligonucleotide, and the results of the genetic crosses with either compatible or incompatible pollen (Figure 5b). Unlike the *ScHT-A* antisense experiment (Figure 4), the RNA interference strategy specifically targeted the *ScHT-B* transcript as no significant variation in the *ScHT-A* mRNA levels could be observed (Figure 5a). Only the transgenic plants with the most reduced *ScHT-B* mRNA level became partially SC (plants #2 and 3), suggesting that a threshold level of *ScHT-B* is necessary to maintain the SI phenotype, and that only the HT-B isoform is involved in GSI.

#### *The ScHT-B gene affects flower longevity and stylar abscission following an incompatible pollination*

One intriguing observation, following an incompatible pollination, was that flowers of *ScHT-B* RNAi plants that had lower levels of *ScHT-B* transcripts, stayed much longer on the plant than control or transgenic plants not affected in *ScHT-B* mRNA levels (plants #4, 7, 9 and 15). Under normal conditions, abscission of unpollinated flowers in *S. chacoense* occurs approximately 5 days after anthesis (Figure 6, unpollinated G4). After a compatible pollination, ovary swelling is clearly detectable 3 days after pollination and stylar abscission occurs approximately 4 days after pollination (Figure 6, V22 × G4). Following an incompatible pollination, abscission is delayed by an average of 24 h when compared to unpollinated flowers (Figure 6, G4 × G4). In *ScHT-B*-suppressed lines, flower abscission was further delayed and only occurred after an 8–9-day period following initial pollination (data not shown). This extended flower longevity phenotype caused by a lower than normal *ScHT-B* mRNA level prompted us to re-examine pollination behaviour with SI pollen under a multiple pollination scheme. In this experiment, *ScHT-B* transgenic plants and untransformed control plants were pollinated on anthesis day, and then on the following 3 days with similar pollen load. Fruit formation was then monitored from day 6 to 12 after pollination. As a control, the transformation host genotype was also repeatedly pollinated. Even after multiple pollination, the untransformed plant (control) and the transgenic plants not affected in *ScHT-B* levels (plants #4, 7, 9 and 15) never sired seeds, indicating that multiple pollination alone, even over a 72-h period, was not sufficient to bypass the SI recognition and rejection system (Figure 5c). For the remaining 12 transgenic plants with altered level of *ScHT-B* mRNA, a total of nine plants were scored as

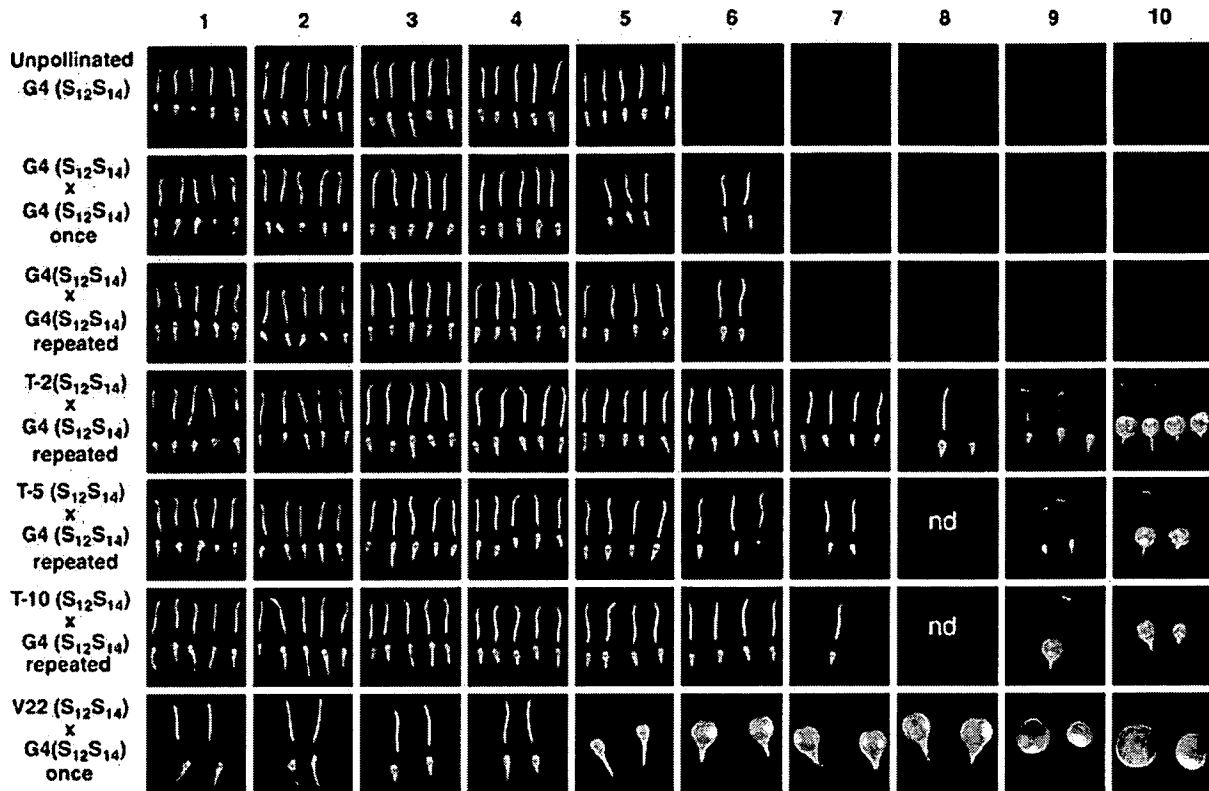


Figure 6. Pistil morphology of *ScHT-B* transgenic and control plants after SI and SC pollination.

Floral and stylar abscission was monitored, from one day after anthesis or from one day after pollination for control plants, and for three transgenic plants (T-2, T-5 and T-10) until day 10. In all cases, pollination was performed with pollen from the G4 line (genotype  $S_{12}S_{14}$ ). Five flowers per plant and per day (except for the V22  $\times$  G4 cross) were hand pollinated on consecutive days and, for the remaining flowers, dissected at the end of the time-course. Only the remaining floral parts at the end of the time-course are displayed. ND, not determined. Once, single pollination. Repeated, consecutive pollination on day 0–2 and if possible on day 3, depending on the corolla closure.

semi-compatible (plants #2, 5, 6, 8, 10–13 and 16) upon repeated pollination with fully incompatible pollen (genotype  $S_{12}S_{14}$ ). Floral and stylar abscission were also monitored, from one day after anthesis or from one day after pollination for control plants and three transgenic plants (T-2, T-5 and T-10) that showed a SC behaviour following self-pollination. Five flowers per plant and per day were hand pollinated on consecutive days in order to collect all samples on the same day (except for the unpollinated control plant and the fully compatible cross V22  $\times$  G4). As such, with the remaining flowers at the end of the 10-day period, the whole time-course was displayed. Pistil morphology for these plants is shown in Figure 6. Transgenic plants, T-2, T-5 and T-10, clearly showed an increased stylar longevity, with turgid styles that appeared receptive to pollination until day 7 or 8, after pollination. Furthermore, stylar abscission from the developing fruit was also delayed, with some styles still attached even after withering (Figure 6, plants T-2 and T-5 on day 9 and 10; plant T-10 on day 9). When compared to a fully compatible cross (V22  $\times$  G4), fruit formation was also delayed in self-pollinated T-2, T-5 and T-10 transgenic plants. These results

confirm the involvement of the *ScHT-B* gene in SI and suggest that it might act through an increased flower receptivity period.

### Discussion

Mechanisms underlying the breakdown of GSI have been recently reviewed and grouped in three broad categories (Stone, 2002). First, loss of SI occurs following the duplication of the *S*-locus and the presence of heterozygous pollen (heteroallelic for the *S*-locus) (Golz *et al.*, 2000). Secondly, mutations affecting either the expression of the *S*-RNase or its activity also lead to a SC phenotype (Royo *et al.*, 1994). Thirdly, mutations not affecting the enzymatic activity of the *S*-RNase have also been described at the genetic level and include many so-called modifier loci. Numerous experiments have demonstrated that although the *S*-RNase is responsible for pollen recognition and rejection in the style (Lee *et al.*, 1994; Matton *et al.*, 1997; Matton *et al.*, 1999; Murfett *et al.*, 1994), other stylar factors are also necessary for the proper expression of the SI phenotype (Ai *et al.*, 1991; Bernatzky *et al.*, 1995; Kondo *et al.*, 2002b; Murfett

et al., 1996). Such factors, often considered as modifier loci, are present in the genetic background of SI plants, unlinked to the *S*-locus, and have often been lost in SC relatives of SI species. Complementation phenomena of the genetic background have been described in *L. hirsutum* where the  $F_1$  population from two independent SC accessions were all SC, while SI offsprings could be recovered in the  $F_2$  generation from these  $F_1$  plants, strengthening the multigenic nature of the gametophytic SI (Ricks and Chetelat, 1991). One such candidate for a modifier gene is the *N. alata* HT gene (McClure et al., 1999). The *NaHT* gene was cloned based on a differential screen between stilar expressed mRNAs from SC *N. plumbaginifolia* and an SC accession of *N. alata* that is defective in S-RNase expression but that is competent to express SI (Murfett et al., 1996). Anti-sense *NaHT* plants with reduced level of the HT protein but with normal levels of S-RNases were either fully or partially SC (McClure et al., 1999). This strongly suggests that the *NaHT* gene is a good candidate for such a modifier factor necessary for the SI reaction to occur. In the present study, we have characterized *NaHT* homologues from four different *Solanum* species, and have focussed our attention on the putative function of the *S. chacoense* HT homologues (*ScHT-A* and *ScHT-B*) in GSI.

Phylogenetic analyses of the isolated *NaHT* homologues clearly demonstrated that two different HT isoforms exist and that isoform B is probably the most closely related to the *NaHT* gene. All the HT proteins shared some common features. Firstly, a highly conserved N-terminal region that is strongly predicted to be a signal peptide. The sequence conservation was high enough to originally derive PCR primer pairs from only the *NaHT* and *ScHT-A<sub>1</sub>* sequences, and amplify both HT isoforms from numerous *Solanum* (this study) and *Lycopersicon* species (Kondo et al., 2002a,b). Secondly, all HT homologues possess a C-terminal region composed of consecutive stretches of asparagine and aspartic acid residues, flanked by conserved cysteines probably involved in disulfide bridges. Although sequence identity is quite variable, ranging from 36 to 92% in the B-isoform group (77–92% when only *Solanum* sequences are considered), the overall structure conservation combined with identical expression pattern would suggest that the *ScHT-B* isoform and the *NaHT* protein are probably true orthologues. Both *ScHT-A*, *ScHT-B* and *NaHT* are almost exclusively stilar-expressed as for the S-RNases, and all are developmentally regulated during pistil maturation (this study and McClure et al., 1999). Interestingly, we found higher expression levels of both *ScHT* and S-RNase genes in the upper style region (Figure 2c), consistent with the pattern of pollen tube arrests that occurs in the top half of the style in *S. chacoense* (Matton et al., 1999). The developmental regulation of S-RNase transcripts accumulation enables the production of selfed progeny in some GSI species when using very young flower buds (bud-

pollination), but is very difficult to achieve in *S. chacoense*. One reason could be the elevated level of both S-RNase and HT transcripts, even 2 days before anthesis, and detectable 3 days before anthesis, combined with a preferential upper style accumulation. One intriguing observation was the differential expression pattern of both S-RNase and HT transcripts following an incompatible pollination compared to an unpollinated or a compatibly pollinated flower (Figure 2d). As the flower ages, S-RNase and HT transcript levels decreases markedly, but low expression levels coincide with reduced fertilization receptivity, and eventually, flower abscission. Surprisingly, S-RNase and *ScHT-A* transcript levels do not decrease following an incompatible pollination (for at least 2 days after pollination, Figure 2d), and this cannot be the result of only stigmatic and transmitting tissue deterioration and death, since this is also induced by a compatible pollination. Furthermore, mechanical wounding or wound hormone treatments had no effect on S-RNase and *ScHT-A* transcript levels. This strongly suggests that the presence of dead pollen tubes or molecules liberated from the arrested pollen tubes, either increase the transcription of these genes, or reduce their mRNA turnovers, ensuring that the S-RNases and HT proteins are still present in sufficient amount to reject newly incoming pollen from incompatible genotypes. The maintenance of high steady-state levels of S-RNases and *ScHT* mRNAs following an initial incompatible pollination, would also lead to a prolonged reproductive barrier, an important issue since flower senescence is retarded following an incompatible pollination (Figure 6, G4 × G4).

In order to determine the role of the *ScHT* genes in SI, functional analysis of *ScHT-A* and *ScHT-B* protein-protein interactions with an S-RNase were tested in the yeast two-hybrid system, and transgenic plants with strongly suppressed levels of both isoforms were generated. Although *ScHT-A* and *ScHT-B* deduced mature proteins have acidic pIs and the S-RNase is basic (predicted pI = 9.25 for S<sub>11</sub>-RNase mature protein), no direct interactions based either on specific or electrostatic attractions could be detected in the two-hybrid system, as no yeast growth could be observed, with or without the predicted signal peptide. Such direct interaction had also not been detected with the purified HT protein from *N. alata*, although in that case, the *NaHT* protein appeared to be unstable in stilar extracts (McClure et al., 1999). Both results suggest that HT proteins and S-RNases do not interact directly.

Recently, correlative evidences for the involvement of the *NaHT* homologues in *Lycopersicon* species have been obtained (Kondo et al., 2002a,b). In the three *Lycopersicon* SI species tested, all expressed functional S-RNases as well as HT-A and HT-B mRNAs. In the seven *Lycopersicon* SC species tested, no or low stilar ribonuclease activity was observed. This alone would most probably be sufficient to explain their SC phenotype, since a threshold level of

S-RNase expression is necessary to confer an SI phenotype (Lee *et al.*, 1994; Matton *et al.*, 1997). Intriguingly, in the seven *Lycopersicon* SC species tested, transcription of the HT-B isoform was either weakly or not detected at all, and the HT-B isoform produced had internal stop codons, while the HT-A isoform was strongly expressed at the mRNA level, although some SC species also produced defective (frame-shifted) HT-A transcripts. Apart from the *N. alata* transgenic antisense lines, no other functional analysis had been made prior to the one presented here. In *N. alata*, plants with reduced levels of the NaHT protein were either fully or partially SC, suggesting that the amount of NaHT protein is important (McClure *et al.*, 1999). In the present study, antisense *ScHT-A* and RNAi *ScHT-B* plants were generated. Figure 4 showed that even with a 15-fold decrease in *ScHT-A* mRNA levels, *ScHT-A* AS plant #9 remained SI. *ScHT-B* mRNA levels in that transgenic line were also affected, although to a lesser extent. This could suggest that a threshold level of *ScHT-B* is sufficient to maintain an SI phenotype. In a second series of experiments, *ScHT-B* suppression was achieved through an RNAi strategy. Unlike the antisense plants, the *ScHT-B* RNAi plants were only affected in *ScHT-B* mRNA expression (Figure 5). Plants with severely reduced *ScHT-B* transcripts became SC and sired seeds upon pollination with pollen from an incompatible genotype. RNAi plant #2 had the most severely reduced *ScHT-B* mRNA levels and consistently set seeds upon self-pollination. RNAi plant #3 had a less stable phenotype, and only sired seeds occasionally. Although at first only two plants became partially SC upon selfing, all the transgenic plants with reduced *ScHT-B* mRNA levels also showed an extended, albeit slightly variable, floral longevity upon pollination with incompatible pollen. This observation led to the hypothesis that the *ScHT-B* gene could be involved in modulating the receptivity period of the flower, perhaps through a control over the abscission of the floral organs. This increase in floral longevity, and in particular in stylar turgescence and receptivity might partially explain the SC phenotype since it could increase the chances of pollen tubes to reach the ovary. To test this, repeated pollination were performed on 3–4 consecutive days, on the 16 RNAi *ScHT-B* transgenic plants and control plants. None of the *ScHT-B* transgenic plants unaffected in *ScHT-B* mRNA expression, or the untransformed control plant, had an extended floral longevity and none were fertilized upon selfing. Of the remaining 12 transgenic plants expressing reduced levels of *ScHT-B* mRNA, nine were able to sire seeds. Fruits formed on these plants were smaller than the ones obtained from a compatible cross, and after 10 days, were comparable in size with fruits produced from a compatible pollination after 6 days (Figure 6, compare the fruits from plants T-2, T-5 and T-10 with the ones from the V22 × G4 cross). These results are entirely consistent with our hypothesis that reduced level of *ScHT-B* mRNA affects

the receptivity period of the flower and that the SC phenotype observed in *ScHT-B* transgenic plants does not result only from the developmentally regulated decrease in both *S-RNase* and *ScHT-B* mRNA levels (Figure 2a,b), since repeated incompatible pollination could not induce fertilization in control or unaffected *ScHT-B* transgenic plants. Furthermore, in *N. alata* HT antisense plants, pollen tube growth in the style is observed even when the S-RNase level is high, although in that case, fertilization and production of fruits could not be observed because the recipient plant used was a sterile hybrid between *N. alata* and *N. plumbaginifolia* and only pollen tube growth in the style was used to score the SI or SC phenotype.

Our results clearly indicate that there is an increase in flower longevity and pollination receptivity, associated with a decrease in *ScHT-B* transcripts. One possibility would be that the HT-B isoform is involved in a pathway regulating floral abscission. Pollination is known to affect the physiological state of the flower. Pollinated flowers (compatible) senesce rapidly compared to unpollinated flowers or those pollinated by incompatible pollen grains in the case of an SI plant (Gilissen, 1977; Singh *et al.*, 1992). Early studies in *Petunia* ovaries showed an increase in polyribosomes activity, 6–12 h after pollination, well before the arrival of the pollen tubes in the ovary (approximately 50 h) (Deurenberg, 1976). Pollination-induced wilting of the corolla can be prevented if the style is removed early after pollination (Gilissen, 1984). These and other results (Stead, 1992) have led to the hypothesis that a pollination-induced signal is transmitted through the pistil and precedes the growing pollen tube. Ethylene has been shown to have a strong effect on flower abscission in solanaceous species (van Doorn, 2002a,b). Furthermore, pollination itself induces ethylene synthesis (Hall and Forsyth, 1967), and it has been shown that in *P. hybrida*, pollination induces two distinct phases of ethylene production in the flower (Singh *et al.*, 1992). The first phase is common to both self- and cross-pollinated flowers and is dependent on pollen-borne ACC (ethylene precursor). The second phase results from *de novo* synthesis of ethylene from the flower and occurs 18 h after a compatible pollination. Following an incompatible pollination, the production of ethylene is delayed to 3 days after pollination (Singh *et al.*, 1992). Since RNAi *ScHT-B* plants showed delayed floral abscission, we tested these plants for alteration in the expression of ethylene-related genes. No differences could be observed in the expression pattern of two genes involved in ethylene biosynthesis (ACC synthase and ACC oxidase), or in ethylene perception and signal transduction (ethylene receptor ETR1 and EIL-3) in *ScHT-B* transgenic plants (data not shown). Since the ACC synthase and the ACC oxidase genes are part of multigene families (at least eight members for the ACC synthase and four members for the ACC oxidase in *S. lycopersicon*) (Llop-Tous *et al.*, 2000), specific probes will need

to be designed for individual members in order to determine if a given isoform is affected in *ScHT-B* mutant background.

From our results, we propose that the *ScHT-B* isoform is involved in at least two phenomena. Firstly, elevated levels of both S-RNases and *ScHT-B* would be necessary for the SI reaction to occur, as determined from McClure's work (McClure *et al.*, 1999) and from the phenotype of the *ScHT-B* RNAi plant T-2. When the *ScHT-B* mRNA levels are below a threshold level, pollen tubes would be able to reach the ovary and effect fertilization. The developmentally regulated decrease in both S-RNase and *ScHT* mRNA levels (Figure 2a,b) would normally lead to an SC phenotype in aged flowers, but is counterbalanced by floral abscission. The increase in both S-RNase and *ScHT* mRNA levels following an incompatible pollination (Figure 2d) would also ensure the maintenance of a strong reproductive barrier over a longer period of time. This could be of importance since flowers pollinated with incompatible pollen last longer by an average of 1 day on the plant than unpollinated flowers (Figure 6, G4 × G4), and the receptivity period for a successful pollination is normally limited to the first 2–3 days after anthesis. Secondly, the *ScHT-B* RNAi transgenic plants display a novel phenotype that includes a longer floral longevity with delayed stylar abscission and, perhaps, more relevant for the SC phenotype of those plants, the persistence of turgid styles, even 9 days after anthesis (Figure 6). This phenotype, observed in plants with reduced levels of *ScHT-B* mRNAs, would enable pollen tube growth in older styles with reduced S-RNase level, pass their normal flower lifespan and pollination receptivity period. As suggested by McClure (McClure *et al.*, 1999), the HT protein could interact directly with pollen tubes and facilitate S-RNase uptake. Our two-hybrid results would support the fact that *ScHT-B* does not interact directly with the S-RNase. Furthermore, if the HT-B protein is involved in S-RNase uptake, a reduced level of HT-B protein would increase the number of pollen tubes not affected by the presence of the S-RNase, enabling fertilization to take place. This is entirely consistent with our results, since repeated pollination in *ScHT-B* RNAi plants increase the percentage of fertilized ovules. Although its mode of action still remains unclear, our data demonstrate a specific role for the HT-B isoform in SI and points towards a role in the control of flower senescence and abscission.

## Experimental procedures

### Plant material and transformation

The diploid ( $2n=2x=24$ ) *S. chacoense* Bitt. SI genotypes used included line 314 ( $S_{11}S_{12}$ ), 582 ( $S_{13}S_{14}$ ), G4 ( $S_{12}S_{14}$ ) and V22 ( $S_{11}S_{13}$ ). Plants were grown in greenhouse with 14–16 h of light per day. Transformation was done as described previously and the

transformation host plant was line G4 (Matton *et al.*, 1997). The *ScHT-A* cDNA was cloned in antisense orientation downstream of a CaMV 35S promoter with doubled enhancers (Skuzeski *et al.*, 1990) and flanked by the nos terminator in the pBIN19 vector (Bevan, 1984). For RNA interference experiments, a new vector was constructed. This new vector, called pDARTH (O'Brien and Matton, unpublished), includes a CaMV 35S promoter with doubled enhancers (Skuzeski *et al.*, 1990), an extended multiple cloning site and a 327-bp intron from a histone deacetylase (*HD2*) gene from *S. chacoense* (our unpublished results). The *ScHT-B* cDNA sequence (324 bp) was cloned in sense and antisense orientation separated by the *HD2* intron.

### Isolation of the *ScHT* cDNAs and PCR amplification of other solanaceous HT genes

The *ScHT-A*, *ScHT-A*<sub>2</sub> and *S*<sub>14</sub>-RNase cDNAs were initially isolated from a pollinated pistil cDNA library using virtual subtraction (Li and Thomas, 1998). In this procedure, genes corresponding to low-expressed mRNA species are preferentially isolated. Because the initial screen was for rare mRNA species expressed in ovary tissues, and since the library also contained cDNAs expressed in styles, genes that were highly expressed in styles but only weakly expressed in ovaries were also recovered. A second screening round with a probe derived from stylar mRNAs, uncovered all of the stylar expressed genes, including the *ScHT-A*, *ScHT-A*<sub>2</sub> and *S*<sub>14</sub>-RNase cDNAs. For the isolation of the *ScHT-B* cDNA and of related sequences in other solanaceous species, three degenerate primers were designed based on the most conserved amino acid sequence of *ScHT-A*, from *S. chacoense* and HT from *N. alata* (McClure *et al.*, 1999). The sequence of the upstream primers (HT-NS1: 5'-TTT CTT TGG TTC TT(A/T) TGA T(A/T)A TAT CAT CA-3'; HT-NS2: 5'-ATA TCA TCA GA(A/G) GTT ATT GC(A/T) AGG GA(A/T) ATG-3') are derived from the predicted signal peptide sequence, and the sequences of the downstream primers (HT-C1: 5'-TCC TTT ATT CAA CCA AT(C/T) TCA TAT TA-3'; HT-C2B: 5'-CAA AAA TAT TAC ATA ATA TTT TGT AGT CG-3') are derived from the C-terminus of the HT protein. The *S. chacoense* HT-B1 isoform was obtained by PCR amplification of cDNAs from a pollinated pistil library while HT isoforms from *S. pinnatisectum*, *S. bulbocastanum* and *S. tuberosum* were obtained by PCR amplification of genomic DNA.

### Isolation and gel blot analysis of RNA and DNA

Total RNA was isolated as described previously (Jones *et al.*, 1985). RNA concentration was determined by measuring its absorbance at 260 nm and verified by agarose gel electrophoresis following ethidium bromide staining. To confirm equal loading of total RNA on RNA-gel blots, a 1-kb fragment of the *S. chacoense* 18S RNA was PCR amplified and used as a probe (Lantin *et al.*, 1999a). Genomic DNA isolation was performed via a modified CTAB extraction method (Reiter *et al.*, 1992) or with the Plant DNeasy kit from Qiagen. DNA-gel blot analysis, including restriction, electrophoresis and capillary transfer to a positively charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Baie D'Urfé, Québec) were performed as described previously (Sambrook *et al.*, 1989). Hybridization of the membrane was performed under high stringency conditions at 65°C as described previously (Church and Gilbert, 1984) for 16–24 h, and following hybridization, the membrane was washed at room temperature twice with 2X SSC/0.1% SDS for 30 min, twice with 1X SSC/0.1% SDS at 50°C for 30 min and twice with 0.1X SSC/0.1% SDS at 55°C for 10 min (1X SSC is 0.15 M

NaCl, 0.015 M sodium citrate, pH 7.0). RNA-gel blot analyses were performed as described in Sambrook *et al.* (1989), following the formaldehyde denaturing protocol. RNAs were capillary transferred to Hybond N+ nylon membranes and cross-linked ( $120 \text{ mJ cm}^{-2}$ ) with a Hoefer UVC 500 UV Crosslinker. Hybridization of the membranes was performed under high stringency conditions at  $45^\circ\text{C}$  in 50% deionized formamide, 5X Denhardt solution, 0.5% SDS,  $200 \mu\text{g ml}^{-1}$  denatured salmon sperm DNA and 6X SSC for 16–24 h. Following hybridization, the membranes were washed at room temperature twice with 2X SSC/0.1% SDS for 30 min, twice with 1X SSC/0.1% SDS at  $50^\circ\text{C}$  for 30 min and twice with 0.1X SSC/0.1% SDS at  $55^\circ\text{C}$  for 10 min. Probes for DNA-gel blot analysis were synthesized from random-labeled isolated DNA inserts (Roche Diagnostic, Laval, Québec) with  $\alpha\text{-}^{32}\text{P}$  dCTP (ICN Biochemicals, Irvine, CA). For RNA-gel blot analyses, cDNA probes were made with  $\alpha\text{-}^{32}\text{P}$  dATP with the Strip-EZ DNA labeling kit (Ambion, Austin, TX) and oligonucleotide probes were labeled with  $\gamma\text{-}^{32}\text{P}$  dATP (Sambrook *et al.*, 1989). The membranes were autoradiographed at  $-85^\circ\text{C}$  with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, Ontario).

#### Site-directed mutagenesis of the *S*<sub>11</sub>-RNase and yeast two-hybrid analysis

A mutated *S*<sub>11</sub>-RNase gene with the conserved His-114 residue (CAT) located in the C3 active site domain was converted to a leucine residue (CTT) by site-directed mutagenesis using the following oligonucleotide (mutated nucleotide is underlined): 5'-CTAAAGCTTGATCCTGCTGT-3' (Altered sites II *in vitro* mutagenesis system, Promega, WI). The original construct contained both the *S*<sub>11</sub> intron and 3' end of the gene, and was expressed in transgenic *S. chacoense* plants (Matton *et al.*, 1997; Matton *et al.*, 1999) under the control of the style specific chitinase promoter (Harikrishna *et al.*, 1996). The spliced His<sup>-</sup> *S*<sub>11</sub>-RNase cDNA was recovered from reversed transcribed style mRNAs, and the coding region corresponding to the mature protein was PCR amplified (Pwo DNA polymerase, Roche Diagnostics, Laval, Québec) and fused in frame with the DNA-binding domain of the GAL4 protein in the pBDGAL4 yeast vector (TRP1 selection marker) (Stratagene, LaJolla, CA). The *S*CHT-A<sub>1</sub> coding region was PCR amplified with or without the predicted signal peptide and inserted in frame with the GAL4 activation domain in the pADGAL4 vector (LEU2 selection marker). For the *S*CHT-B construct, only the coding region without the predicted signal peptide was inserted in frame with the GAL4 activation domain in the pADGAL4 vector. Integrity of the DNA constructs was verified by sequencing. The constructs were transformed sequentially in the yeast strain PJ69-4A (James *et al.*, 1996) and selected through their ability to grow on Trp<sup>-</sup> and Leu<sup>-</sup> media. Protein-protein interaction assays were performed on media lacking Trp, Leu and His and on media lacking Trp, Leu and Ade.

#### Acknowledgements

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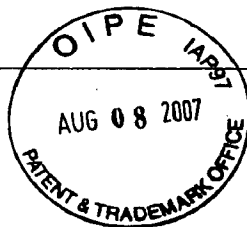
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GenBank accession numbers: *ScHT-A*<sub>1</sub> (AF442139), *ScHT-A*<sub>2</sub> (AF442140), *ScHT-B*<sub>1</sub> (AF442141), *StHT-A*<sub>1</sub> (AF442142), *SbHT-B*<sub>1</sub> (AF442143), *SbHT-B*<sub>2</sub> (AF442144), *SpHT-B*<sub>1</sub> (AF442145), *SpHT-B*<sub>2</sub> (AF442146), *LpHT-A*<sub>1</sub> (AB066582), *LpHT-B*<sub>1</sub> (AB066583), and *S*<sub>14</sub>-RNase (AF232304).





Patent  
Attorney's Docket No. 021565-060

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of	)	
Peter Waterhouse et al.	)	Group Art Unit: 1635
Application No.: 09/287,632	)	Examiner: ZARA JANE
Filed: April 7, 1999	)	Confirmation No.: 6526
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED	)	
PHENOTYPES	)	

**Declaration of Dr. Elizabeth Salisbury Dennis under 37 C.F.R. section 1.132**

I, Elizabeth Salisbury Dennis, hereby declare that:

1. I am a citizen of Australia. I received a Ph. D. degree in 1968 from the University of Sydney, Australia. I am an author or co-author of approximately 200 peer-reviewed research papers in the field of plant molecular biology. A copy of my curriculum vitae is attached as Exhibit I.
2. My present position is Chief Research Scientist/CSIRO Fellow in the Division of Plant Industry of the Commonwealth Scientific and Industrial Research Organisation ("CSIRO"). My present work at CSIRO includes management and supervision of research projects in plant molecular biology, including research projects on gene silencing or that use gene silencing techniques.
3. I am very experienced in the field of molecular biology, including the field of post-transcriptional gene silencing and have authored and co-authored scientific publications in this field as listed in my curriculum vitae. I, together with others working with me, have incorporated introns in genetic constructs which were expressed in plant cells, and my research group was one of the first to do so. I am personally experienced with and familiar with the use of introns.
4. I have read US Patent Application No. 09/287,632 (the "Application") including the presently pending claims and the Office Action dated 8 February 2007 issued by the United States Patent and Trademark Office in connection with the Application.
5. I note that in the Office Action the Examiner alleged that "The claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention."
6. It is my understanding that the Examiner believes that the specification, claims

and the art do not adequately describe the distinguishing features or attributes shared by the members of the claimed genus of DNA constructs for gene silencing comprising the recited sense and antisense nucleotide sequences and further comprising an intron sequence, whereby any intronic sequence is inserted anywhere in the chimeric DNA, and whereby the DNA construct provides for the function claimed, namely reducing the phenotypic expression of a nucleic of interest in any plant cell or in any isolated eukaryotic cell.

7. I have been informed, and confirmed for myself, that the claimed subject matter recites that the intron is in the (transcribed) DNA region (for example claim 1, line 15), not "anywhere in the chimeric DNA" as mentioned by the Examiner in the Office Action, page 4, line 6.
8. The Examiner also asserts that the Application provides only two examples of chimeric constructs which contain an intron. From this, I understand that the Examiner believes that the Application does not describe a sufficient number of different possibilities for the chimeric dsRNA constructs claimed to allow a person skilled in the art to conclude that the inventors were in possession of the genus as broadly claimed.
9. In my view a person skilled in the art most relevant to the claimed subject matter is a person with a PhD in the field of molecular biology of eukaryotes with at least three years post doctoral research experience.
10. I disagree with the Examiner for the following reasons.
11. The Application teaches at least on page 23, lines 5 to 15 that the chimeric DNA constructs of the invention may comprise an intron in the transcribed region that encodes the double-stranded RNA molecule, that the inclusion of the intron enhances the efficiency of reduction of expression of the target nucleic acid of interest, and that the intron is preferably but not necessarily located in the spacer region. The Application also teaches on page 23, lines 13 to 15 that the intron in a "particularly preferred embodiment" is the *Flaveria trinervia* pyruvate orthophosphate dikinase 2 intron 2 as used in Example 6. It is clear to me that the teaching of the Application is not limited to this specific example or the particular intron. It is my firm opinion that on reading the Application, the person skilled in the art would have readily and immediately understood that the exemplified intron could be substituted for other introns well known in the art, and that other introns would function in the same manner. Moreover, the person skilled in the art would also have understood that the precise location of the intron in the transcribed region of the chimeric gene was not critical, given the fact that the intron would be spliced out of the transcribed RNA, yielding the RNA molecule comprising the double stranded RNA region. It is obvious to me and would be clear to the skilled person that splicing out of the intron would lead to the same RNA molecule irrespective of the position of the intron in the transcribed DNA region.
12. At the filing date of the application, numerous introns from a wide range of

eukaryotic organisms and genes were well known in the art and were well characterised. Furthermore, the mechanism of splicing of introns from primary transcripts was widely understood in the art. To evidence this, I refer to excerpts from a textbook widely used by post-graduate students during the years prior to 1998. These excerpts (Exhibit 2) describe the structure and function of introns including their splicing from primary RNA transcripts. (Molecular Biology of the Cell, second edition, pages 102; 486-487, 532-535.)

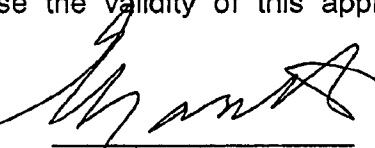
13. As commonly taught in the art, as evidenced in Exhibit 2, introns were first discovered and reported in 1977 and in the following years were shown to be present in many eukaryotic genes. In most eukaryotic genes, the protein encoding sequences (exons) were found to be interrupted by non-coding sequences (introns). It was known that to produce a protein, a gene encoding the protein was first transcribed into an RNA molecule (the primary transcript) by transcription of both the exons and introns. Before or during transport of the RNA molecule from the nucleus to the cytoplasm of the cell, a complex of RNA processing enzymes removes the intron sequences, thereby producing a shorter, mature RNA molecule, which is then ultimately translated in the cytoplasm. This process is commonly called intron splicing. The size of introns is commonly in the range of about 80 nucleotides to about 10 kilobases or more, and the internal sequence of introns is generally highly variable and not conserved. The only highly conserved sequences between introns are those required for intron removal. Consensus sequences have been identified at both ends of almost all introns that are important in intron splicing. These conserved sequences are located at the boundaries of the intron sequence at the 5' end (donor site) and at the 3' end (acceptor site). The pathway by which the introns are removed has also been elucidated and is illustrated for example in Exhibit 2, page 534, demonstrating the involvement of a spliceosome which contains proteins as well as small nuclear RNA molecules, conserved in eukaryotic species.
14. At the filing date of the application, a person of ordinary skill in the art would have been well aware of many different introns existed in eukaryote genes, that these introns were removed from the primary transcripts by a universally conserved RNA splicing pathway, and that the important structural features for the removal of introns from primary transcripts were highly conserved between different introns. Thus, a person of ordinary skill in the art would have appreciated the interchangeability of the intron that is specifically exemplified in the Application for any other intron sequence. Accordingly, given what was known at the filing date of the Application, a person of ordinary skill in the art would have appreciated from reading the Application that the chimeric DNA molecules of the invention could comprise any of a variety of introns and would have known of a wide variety of introns that could be used in place of the intron used in the example.
15. Furthermore, it is my opinion that the person skilled in the art when reading the Application would have realized that the exact location of the intron in the transcribed region of the chimeric gene encoding the dsRNA gene silencing molecules was not critical for the obtained gene silencing effect, as such intron

sequences are removed from the primary transcripts during splicing, and that the resultant spliced dsRNA molecule would be the same irrespective of the exact position of the intron in the transcribed region. Therefore, the chimeric genes would be expected to be functional irrespective of the position of the intron in the transcribed region.

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16. Therefore it is my firm opinion that when the person skilled in the art is taught by the present application to include an intron in the chimeric DNA, it would have been immediately clear to such person that the teaching of the Application is not limited to the specific intron sequence which was exemplified, nor to the preferred location which was exemplified.
17. In support of my opinion expressed above, I am aware that many scientists have, subsequently, successfully used any of a variety of intron sequences in the transcribed region of the chimeric DNA by following the teaching of the Application as reported in Smith et al., (2000) (Exhibit 3). Examples of a number of such reports are attached as Exhibits 4 to 12. To me, this is clear and obvious evidence indicating that the teaching of the Application, in combination with the known art concerning introns, described the invention in sufficient manner to allow numerous others, skilled in the art, to make and use the invention.
18. In conclusion, it is therefore my opinion that a person of ordinary skill in the art would have concluded, judging at the filing date of the Application, that the Application adequately described all of the necessary features of the claimed subject matter in a manner sufficient to convey that the inventors were in possession of the invention as presently claimed.

I also declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of this application or any patent issued thereon.

August 6, 2007  
Date

  
Elizabeth Salisbury Dennis

# CURRICULUM VITAE

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**Name:** Elizabeth Salisbury DENNIS  
**Date of Birth:** 10 December 1943  
**Place of Birth:** Sydney, Australia  
**Nationality:** Australian  
**Address:** CSIRO Plant Industry, Cnr Barry Drive and Clunies Ross Street, Black Mountain, Canberra, ACT 2601  
**Telephone:** W. (02) 6246 5061  
**Facsimile:** (02) 6246 5000  
**Email:** liz.dennis@csiro.au

## Education:

1964 B.Sc. (Hons. 1st Class) Sydney University  
1968 Ph.D. Sydney University  
Thesis entitled: "Studies on the *Bacillus subtilis* Genome"  
Supervisor Dr R.G. Wake

## Academic Honours:

1980 Awarded Whiteley Prize for best zoological book on Papua New Guinea for "A Handbook of New Guinea Rodents" by J.I. Menzies and E.S. Dennis  
1987 Elected Fellow of the Australian Academy of Technological Sciences and Engineering  
1988 Pharmacia LKB/Biotechnology Medal of the Australian Biochemical Society for contributions to Biochemical Research  
1995 Elected Fellow of the Australian Academy of Science  
1997 Avon "Spirit of Achievement" Award  
1998 Awarded Lemberg Medal of the Australian Society of Biochemistry & Molecular Biology  
2000 Awarded the Inaugural Prime Minister's Prize for Science with Dr Jim Peacock  
2002 Fellow of the American Society for the advancement of Science

## Scholarships & Fellowships:

1961-64 Commonwealth Scholarship/Women's College Scholarship, University of Sydney  
1965-68 Commonwealth Post Graduate Scholarship  
1968-70 Post Doctoral Fellow, Anna Fuller Fund  
1982-83 Fulbright Fellowship, Senior Scholar

## Positions Held:

1968-70 Post Doctoral Fellow in laboratory of Dr Julius Marmur, Albert Einstein College of Medicine, New York, USA  
1970-72 Lecturer in Microbiology then Lecturer in Biochemistry, University of Papua New Guinea  
1972-74 Research Scientist, CSIRO Division of Plant Industry, Canberra  
1974-76 Senior Lecturer in Biochemistry, University of Papua New Guinea  
1976-present Senior Research Scientist, Principal Research Scientist, Senior Principal Research Scientist, Chief Research Scientist, CSIRO Fellow - CSIRO Plant Industry, Canberra  
1982-83 Visiting Fellow, Biochemistry Department, Stanford University, Laboratory of Dr Paul Berg

**Societies:**

Genetics Society of Australia (Committee Member 1978-1979)  
Australian Society for Biochemistry and Molecular Biology  
(President-Elect 1991, President 1992-93). Past President 1994.  
International Society of Plant Molecular Biology (Board of Directors, 1990-93)  
American Society of Plant Physiologists

**Association with Universities:**

Visiting Fellow: Australian National University  
Adjunct Professor: Australian National University

**Invited Speaker in International Meetings:**

1974	U.S. Australia Meeting, "The Eukaryote Chromosome", Canberra, Australia
1981	XIII International Botanical Congress, Sydney, Symposium speaker
1982	Gordon Conference, Plant Molecular Biology, U.S.A.
1983	XV International Congress of Genetics (India) Agrigenetics Research Associates Meeting, Cancun, Mexico
1984	Arco-UCLA-Meeting, Cellular and Molecular Biology of Plant Stress, Keystone, U.S.A.
1985	1st International Congress of Plant Molecular Biology, Georgia, U.S.A., Symposium speaker Gordon Conference, Plant Molecular Biology, U.S.A. Agrigenetics Research Associates Meeting, Florida, U.S.A. German-Australia Meeting, "Biotechnology of Cereals", Germany
1986	Cold Spring Harbor Meeting on "Transposable Elements", Cold Spring Harbor, U.S.A.
1987	NATO Meeting, "Plant Molecular Biology, 1987", Copenhagen, Denmark FASEB Meeting, "Plant Gene Expression", Copper Mountain, U.S.A. International Meeting on "Plant Transposable Elements", Madison, Wisconsin, U.S.A. Agrigenetics Research Associates Meeting, Wisconsin, U.S.A.
1988	2nd International Congress of Plant Molecular Biology, Jerusalem, Symposium speaker
1989	Royal Society Meeting, "DNA Methylation and Gene Activity", London, U.K. 10th EMBO/EMBL Symposium, "Molecular Communication in Higher Plants", Heidelberg, F.R. Germany
1990	United Nations Industrial Development Organization International Centre for Genetic Engineering and Biotechnology, "Molecular & Genetic Approaches to Plant Stress" Delhi, India French-Australian Meeting, "Plant Molecular Biology", Versailles, France
1991	Keystone Symposium "Genetic Dissection of Plant Cell Processes", Keystone, USA. US:Australian Workshop, "Flowering", Hawaii, U.S.A. Society for Experimental Biology, "Biochemistry & Molecular Biology of Inducible Enzymes" Birmingham, U.K. 1st Queenstown Molecular Biology Meeting: Queenstown, New Zealand 3rd International Congress of Plant Molecular Biology, Tuscon, Arizona, U.S.A. - Symposium speaker
1992	International Workshop "Molecular Control of Flower Development and Plant Reproduction", Amsterdam, The Netherlands
1993	XV International Botanical Congress, Yokohama, Japan, Symposium speaker "Frontiers of Gibberellin Research", Tokyo, Japan
1994	USA/Australian Workshop "Plants for the Future" Cairns, Australia
1995	8th International Congress on Isozymes, Brisbane, Australia 15th International on Plant Growth Regulators, Minneapolis, USA, Symposium speaker 3rd NIAR/COE Symposium "Dynamics of Plant Genome Structure and Function", Tsukuba, Japan 10th FAOBMB Congress, Sydney, Australia, Symposium speaker
1997	Gordon Conference January 1997 "Low Temperature Effects in Plants", Ventura, California
1998	Molecular Physiology II "Genetic Engineering of Plants for Hostile Environments", Rothamstead, UK, Dec.

1999	"Genetic Control of Flowering" Symposium, Tsukuba, Japan, Nov.
2000	AAAS 2000 Genome Seminar, Washington, DC.
2001	John Innes Institute UK, EMBO Workshop - "The Molecular Basis of the Floral Transition".
2002	17 <sup>th</sup> Long Ashton International Symposium, UK "New Frontiers in Plant Development: from Genes to Phenotype".
2003	XIX International Congress of Genetics – Melbourne August First International Symposium on Rice Functional Genomics – Shanghai China Frontiers in Plant Development Biology – Tokyo Japan Presentation to students at Okayama University
2004	International Conference on Arabidopsis, Germany, July International Plant Growth Substances, Canberra, August Wheat conference – Fourth International Crop Science Congress, Brisbane, August

#### **R&D Achievements in Collaborations with Industry:**

Under Agrigenetics Corporation funding was part of a team which developed plant promoters which respond to anaerobic conditions and give high level expression. This resulted in patents which have been granted in the USA.

Extended this work with Wheat Industry Research Council Funding to develop a monocot promoter with high levels of expression for use in cereal transformation. This has also been patented.

Led a team funded by Gene Shears Pty Ltd to use ribozymes to develop a system for male sterility.

Played a role in the collaboration with Cotton Industry in developing genetically engineering cotton for use in Australia.

Led a team to develop a genetically engineered eucalypts for plantations. GIRD grant in collaboration with CSIRO, Forestry & APM, ANM, North Eucalypt, Technologies & Kimberly Clarke.

Program Leader in GrainGene in association with GRDC, AWB and CSIRO PI (Program 1).

Program Leader - Rice CRC (Program 2)

Leader of projects under CSIRO/Bayer

Board Member - Australian Genome Research Facility

Research Director - New South Wales Centre for Agricultural Genomics

#### **Government Committees:**

1986-91	Biotechnology Advisory Committee of the IR & D Board, Dept of Industry, Technology and Commerce, Australian Government
1985-87	Post Doctoral Awards Committee of CSIRO
1988	Member Australian Government Biotechnology Mission to the European Community
1989	Australian Government observer at European Community Biotechnology Action Program Meeting, "Plant Biotechnology in the European Community", Bad Honnef, West Germany
1990	Ad Hoc Genome Committee, DITAC "Plant Molecular Biology" Co-organizer French-Australian Meeting,
1993-94	Chairman, Multinational Coordinated Arabidopsis Genome Research Project
1994	Rice Biotechnology Working Group, Overseas Development Agency, UK Government

**Journals:**

1987-93 Editor "Advances in Plant Gene Research", Springer, Vienna, Austria  
1990-93 Editor "Genetical Research", Cambridge, U.K.  
1990-99 Editor "The Plant Journal", Blackwell & Bios, U.K., Foundation Editor  
Editorial Board "Molecular Breeding", Kluwer, Netherlands  
Editorial Board "Current Opinion in Plant Biology"

**Research Interests:**

Molecular basis of plant development particularly the initiation of flowering  
Plant gene regulation  
Molecular basis of plant response to stress



## PUBLICATIONS - E.S. DENNIS

*Also published as E.S. Goldring*

1. **Dennis, E.S.** and Wake, R.G. (1965)  
Clotting of milk by proteolytic enzymes  
*Biochim. Biophys. Acta* 97, 159-162
2. **Dennis, E.S.** and Wake, R.G. (1966)  
Autoradiography of the *Bacillus subtilis* chromosome  
*Journal of Molecular Biology*, 15, 435-439
3. **Dennis, E.S.** and Wake, R.G. (1968)  
The *Bacillus subtilis* genome: studies of its size and structure  
In "Replication and Recombination of Genetic Material", 61-70  
(Eds. Peacock, W.J. and Brock, R.D.)  
Australian Academy of Science, Canberra
4. **Goldring, E.S.** and Wake, R.G. (1968)  
A comparison of the segregation of chromosomes within microcolonies  
developing from single *Bacillus subtilis* and *Bacillus megaterium* spores  
*Journal of Molecular Biology*, 35, 647-650
5. Grossman, L.I., **Goldring, E.S.** and Marmur, J. (1969)  
Preferential synthesis of yeast mitochondrial DNA in the absence of protein synthesis  
*Journal of Molecular Biology*, 46, 367-376
6. **Goldring, E.S.**, Grossman, L.I., Krupnick, D., Cryer, D.R. and Marmur, J.  
(1970)  
The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid  
during induction of petites with ethidium bromide  
*Journal of Molecular Biology*, 52, 323-335
7. **Goldring, E.S.**, Grossman, L.I. and Marmur, J. (1971)  
Petite mutation in yeast. II. The isolation of mutants containing  
mitochondrial deoxyribonucleic acid of reduced size.  
*Journal of Bacteriology*, 107, 377-381
8. Grossman, L.I., Cryer, D.R., **Goldring, E.S.** and Marmur, J. (1971)  
The petite mutation in yeast. III. Nearest-neighbour analysis of  
mitochondrial DNA from normal and mutant cells  
*Journal of Molecular Biology*, 62, 565
9. Peacock, W.J., Brutlag, D., **Goldring, E.S.**, Appels, R., Hinton, C.W. and  
Lindsley, D.L. (1974)  
The organization of highly repeated DNA sequences in *Drosophila*  
*melanogaster* chromosomes  
Cold Spring Harbor Symposium on Quantitative Biology. 38, 405-416
10. **Goldring, E.S.**, Brutlag, D.L. and Peacock, W.J. (1975)  
Arrangement of the highly repeated DNA of *Drosophila melanogaster*  
In "The Eukaryote Chromosome", 47-59  
(Eds. Peacock, W.J. and Brock, R.D.)  
Austalian National University Press, Canberra
11. Maddocks, I., Anders, E.M. and **Dennis, E.S.** (1976)  
Donovanosis in Papua New Guinea  
*British Journal of Venereal Diseases*, 52, 190-196

12. Price, M.A., Anders, E.M., Anders, R.F., Russell, D.A. and **Dennis, E.S.** (1976)  
Cell-mediated immunologic status of healthy members of families with a history of leprosy  
*International Journal of Leprosy*, 43, 307-313
13. Peacock, W.J., Appels, R., Gerlach, W.L. and **Dennis, E.S.** (1976)  
DNA sequence probes - a new technique in plant breeding  
CSIRO Division of Plant Industry Annual Report 1976, 41-45
14. Brutlag, D., Appels, R., **Dennis, E.S.** and Peacock, W.J. (1977)  
Highly repeated DNA in *Drosophila melanogaster*  
*Journal of Molecular Biology*, 112, 31-47
15. **Goldring, E.S.** and Peacock, W.J. (1977)  
Intramolecular heterogeneity of mitochondrial DNA of *Drosophila melanogaster*  
*Journal of Cell Biology*, 73, 279-286
16. **Dennis, E.** and Menzies, J.I. (1978)  
Systematics and chromosomes of New Guinea *Rattus*  
*Australian Journal of Zoology*, 26, 197-206
17. Peacock, W.J., Lohe, A.R., Gerlach, W.L., Dunsmuir, P., **Dennis, E.S.** and Appels, R. (1978)  
Fine structure and evolution of DNA in heterochromatin  
*Cold Spring Harbor Symposia on Quantitative Biology*, 42, 1121-1135
18. Gerlach, W.L., Appels, R., **Dennis, E.S.** and Peacock, W.J. (1978)  
Evolution and analysis of wheat genomes using highly repeated DNA sequences  
Proc. 5th Int. Wheat Genetics Symposium, New Delhi, 1978, 1, 81-91
19. **Dennis, E.** and Menzies, J.I. (1979)  
A chromo A chromosomal and morphometric study of Papuan tree rats  
*Pogonomys* and *Chiruromys* (Rodentia, Muridae)  
*Journal Zoology. (Lond.)* 189, 315-332
20. Schwinghamer, E.A. and **Dennis, E.S.** (1979)  
Electron microscopic evidence for a multimeric system of plasmids in fast-growing *Rhizobium* spp.  
*Australian Journal of Biological Science*, 32, 651-662
21. Menzies, J.I. and **Dennis, E.S.** (1979)  
"Handbook of New Guinea Rodents"  
(Bernice P. Bishop Museum: Hawaii)  
Wau Ecology Institute Handbook 6  
(Wau Ecology Institute: Papua New Guinea)
22. Appels, R., Gerlach, W.L., **Dennis, E.S.**, Swift, H. and Peacock, W.J. (1980)  
Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals  
*Chromosoma (Berl.)* 78, 293-311
23. **Dennis, E.S.**, Gerlach, W.L. and Peacock, W.J. (1980)  
Identical polypyrimidine-polypurine satellite DNAs in wheat and barley  
*Heredity*, 44, 349-366
24. **Dennis, E.S.**, Dunsmuir, P. and Peacock, W.J. (1980)  
Segmental amplification in a satellite DNA: Restriction enzyme analysis of the major satellite of *Macropus rufogriseus*  
*Chromosoma (Berl.)* 79, 179-198

25. Peacock, W.J., **Dennis, E.S.**, Hilliker, A.J. and Pryor, A.J. (1980)  
Differentiation of heterochromatin  
In "Evolution and Speciation: Essays in Honour of M.J.D. White", 78-100  
(Eds. Atchley, W.R. and Woodruff, D.S.) Cambridge University Press, New York
26. Peacock, W.J., Gerlach, W.L. and **Dennis, E.S.** (1981)  
Molecular aspects of wheat evolution: Repeated DNA sequences  
In "Wheat Science - Today and Tomorrow", 41-60  
(Eds. Evans, L.T. and Peacock, W.J.) Cambridge University Press, Cambridge
27. **Dennis, E.S.**, Peacock, W.J., White, M.J.D., Appels, R. and Contreras, N. (1981)  
Cytogenetics of the parthenogenetic grasshopper *Warramaba virgo* and its bisexual relatives. VII: Evidence from repeated DNA sequences for a dual origin of *W. virgo*  
*Chromosoma (Berl.)*, 82, 453-469
28. Peacock, W.J., **Dennis, E.S.** and Gerlach, W.L. (1981)  
Satellite DNA - change and stability  
In "Chromosomes Today", 7, 30-41  
Proc. 7th Int. Chromosome Conf., Oxford, 1980  
(Eds. Bennett, M.D., Bobrow, M. and Hewitt, G.) George Allen and Unwin, London
29. Peacock, W.J., **Dennis, E.S.**, Elizur, A. and Calaby, J.H. (1981)  
Repeated DNA sequences and kangaroo phylogeny  
*Australian Journal of Biological Science*, 34, 325-340
30. Peacock, W.J., **Dennis, E.S.**, Rhoades, M.M. and Pryor, A.J. (1981)  
Highly repeated DNA sequence limited to knob heterochromatin in maize  
*Proceedings of the National Academy of Sciences, USA*, 78, 4490-4494
31. Appels, R., **Dennis, E.S.**, Smyth, D.R. and Peacock, W.J. (1981)  
Two repeated DNA sequences from the heterochromatic regions of rye (*Secale cereale*)  
chromosomes  
*Chromosoma (Berl.)* 84, 265-277
32. Elizur, A., **Dennis, E.S.** and Peacock, W.J. (1982)  
Satellite DNA sequences in the red kangaroo (*Macropus rufus*)  
*Australian Journal of Biological Science*, 35, 313-325
33. Gerlach, W.L., Pryor, A.J., **Dennis, E.S.**, Ferl, R.J., Sachs, M.M. and Peacock, W.J. (1982)  
cDNA cloning and induction of the alcohol dehydrogenase gene (*Adh1*) of maize  
*Proceedings of the National Academy of Sciences, USA*, 79, 2981-2985
34. Peacock, W.J., **Dennis, E.S.** and Gerlach, W.L. (1982)  
DNA sequence changes and speciation  
In "Mechanisms of Speciation", 123-142  
(Ed. Barigozzi, C.) Alan R. Liss, Inc., New York
35. White, M.J.D., **Dennis, E.S.**, Honeycutt, R.L., Contreras, N. and Peacock, W.J. (1982)  
Cytogenetics of the parthenogenetic grasshopper *Warramaba virgo* and its bisexual relatives. IX. The ribosomal RNA cistrons  
*Chromosoma (Berl.)* 85, 181-199

36. Sachs, M.M., Lorz, H., **Dennis, E.S.**, Elizur, A., Ferl, R.J., Gerlach, W.L., Pryor, A.J. and Peacock, W.J. (1982)  
Molecular genetic analysis of the maize anaerobic response  
In "Maize for Biological Research", 139-144(Ed. Sheridan, W.F.)  
Plant Molecular Biology Association, Charlottesville, VA
37. Gerlach, W.L., Lörz, H., Sachs, M.M., Llewellyn, D., Pryor, A.J., **Dennis, E.S.** and Peacock, W.J. (1983)  
The alcohol dehydrogenase genes of maize: A potential gene transfer system in plants  
In "Manipulation and Expression of Genes in Eukaryotes", 213-220  
Proc. Int. Conf. Monash University, 1982  
(Eds. Nagley, P., Linnane, A.W., Peacock, W.J. and Pateman, J.A.)  
Academic Press Australia
38. Sachs, M.M., Peacock, W.J., **Dennis, E.S.** and Gerlach, W.L. (1983)  
Maize *Ac/Ds* controlling elements.  
A molecular viewpoint. *Maydica* 28, 289-301
39. **Dennis, E.S.** and Peacock, W.J. (1983)  
Knob heterochromatin DNA: organization and evolution  
In "Genetics: New Frontiers. Vol. I Genetic Organization, Function and Regulation", 247-253  
Proc. 15th Genetics Congr., New Delhi, 1983  
(Eds. Chopra, V.L., Joshi, B.C., Sharma, R.P. and Bansal, H.C.)  
Oxford and IBH Publishing Co., New Delhi
40. Gerlach, W.L., **Dennis, E.S.** and Peacock, W.J. (1983)  
Molecular cytogenetics of wheat  
In "Cytogenetics of Crop Plants", 191-212  
(Eds. Swaminathan, M.S., Gupta, P.K. and Sinha, U.)  
Macmillan India Limited
41. Peacock, W.J., **Dennis, E.S.**, Gerlach, W.L., Llewellyn, D., Lorz, H., Pryor, A.J., Sachs, M.M., Schwartz, D. and Sutton, W.D. (1983)  
Gene transfer in maize: controlling elements and the alcohol dehydrogenase genes  
In "Advances in Gene Technology: Molecular Genetics of Plants and Animals", 311-325  
Miami Winter Symp. 20  
(Eds. Downey, K., Voellmy, R.W., Ahmad, F. and Schultz, J.)  
Academic Press: New York  
Reprinted in "Genetic Manipulation: Impact on Man and Society", 115-126 (1984)  
(Eds. Arber, W., Illmensee, K., Peacock, W.J. and Starlinger, P.)  
Cambridge University Press, Cambridge
42. **Dennis, E.S.**, Gerlach, W.L., Pryor, A.J., Bennetzen, J.L., Inglis, A., Llewellyn, D., Sachs, M.M., Ferl, R.J. and Peacock, W.J. (1984)  
Molecular analysis of the alcohol dehydrogenase (*Adh1*) gene of maize  
*Nucleic Acids Research*, 12, 3983-4000
43. Howard, E.A. and **Dennis, E.S.** (1984)  
Transposable elements in maize - the activator-dissociation (Ac-Ds) system  
*Australian Journal of Biological Science*, 37, 307-314
44. **Dennis, E.S.**, Hohn, B., Hohn, Th., King, P.J., Schell, J. and Verma, D.P.S. (Eds.) (1984)  
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Springer-Verlag, Wien

45. Peacock, W.J., **Dennis, E.S.**, Gerlach, W.L., Sachs, M.M. and Schwartz, D. (1984)  
Insertion and excision of *Ds* controlling elements in maize  
Cold Spring Harbor Symposia on Quantitative Biology, 49, 347-354
46. **Dennis, E.S.**, Waldron, J.C. and Peacock, W.J. (1984)  
Genetic engineering and plant breeding  
In "Pollination" 84, 98-101  
Proc. Symposium, University of Melbourne, 1984 (Eds. Williams, E.G. and Knox, R.S.)  
University of Melbourne, Parkville
47. Gerlach, W.L., **Dennis, E.S.** and Peacock, W.J. (1984)  
Controlling elements in maize  
Aust. CSIRO Divisional Annual Report 1983-84, 20-24
48. **Dennis, E.S.** and Peacock, W.J. (1984)  
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Journal of Molecular Evolution, 20, 341-350
49. **Dennis, E.S.**, Sachs, M.M., Gerlach, W.L. Finnegan, E.J. and Peacock, W.J. (1985)  
Molecular analysis of the alcohol dehydrogenase 2 (*Adh2*) gene of maize  
Nucleic Acids Research, 13, 727-743
50. Llewellyn, D., **Dennis, E.S.** and Peacock, W.J. (1985)  
The alcohol dehydrogenase genes of maize: Expression studies in tobacco cells  
In "Molecular Form and Function of the Plant Genome", 593-607  
Proc. NATO Adv. Study Inst.-FEBS Adv. Course on Mol. Form and Function on the Plant Genome, Renesse, 1984  
(Eds. Vloten-Doting, L. van, Groot, G.S.P. and Hall, T.C.)  
Plenum Publishing Corporation, New York
51. Hohn, B. and **Dennis, E.S.** (Eds.) (1985)  
"Genetic Flux in Plants", 253  
"Plant Gene Research -Basic Knowledge and Application", 2  
(Eds. **Dennis, E.S.**, Hohn, B., Hohn, Th., King, P.J., Schell, J. and Verma, D.P.S.)  
Springer-Verlag, Wien
52. **Dennis, E.S.** and Berg, P. (1985)  
Transcription from a plant gene promoter in animal cells  
Nucleic Acids Research, 13, 7945-7957
53. Howard, E.A., Danna, K.J., **Dennis, E.S.** and Peacock, W.J. (1985)  
Transient expression in maize protoplasts  
In "Plant Genetics", 225-234  
Proc. 3rd Annu. ARCO Plant Cell Res. Inst. - UCLA Symp.on Plant Biology, Keystone, Co., 1985  
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54. Sachs, M.M., **Dennis, E.S.**, Ellis, J., Finnegan, E.J., Gerlach, W.L., Llewellyn, D. and Peacock, W.J. (1985)  
*Adh1* and *Adh2*: Two genes involved in the maize anaerobic response  
In "Cellular and Molecular Biology of Plant Stress", 217-226  
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55. Brettell, R.I.S., **Dennis, E.S.**, Scowcroft, W.R. and Peacock, W.J. (1986)  
Molecular analysis of a somaclonal mutant of maize alcohol  
dehydrogenase  
Molecular and General Genetics, 202, 235-239
56. Peacock, W.J. and **Dennis, E.S.** (1986)  
Plant gene engineering and plant agriculture  
In "Biotechnology: Potentials and Limitations", 223-239  
Report of the Dahlem Workshop, Berlin, 1985. Life Sciences Research  
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(Ed. Silver, S.)  
Springer-Verlag, Berlin
57. **Dennis, E.S.**, Gerlach, W.L. and Peacock, W.J. (1986)  
Excision of the *Ds* controlling element from the *Adh1* gene of maize  
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58. Sachs, M.M., **Dennis, E.S.**, Gerlach, W.L. and Peacock, W.J. (1986)  
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poly(A) addition polymorphisms  
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59. Landsmann, J., **Dennis, E.S.**, Higgins, T.J.V., Appleby, C.A., Kortt, A.A. and  
Peacock, W.J. (1986)  
Common evolutionary origin of legume and non-legume plant haemoglobins  
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60. Gerlach, W.L., Sachs, M.M., Llewellyn, D.J., Finnegan, E.J. and **Dennis, E.S.** (1986)  
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61. Appleby, C.A., Bogusz, D., **Dennis, E.S.**, Dudman, W.F., Fleming, A.I., Higgins, T.J., Kortt,  
A.A., Landsmann, J., Peacock, W.J., Tjepkema, J.D., Trinick, M.J., Wittenberg, B.A. and  
Wittenberg, J.B. (1986)  
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Australian Institute of Agricultural Science, Parkville, Vic
62. Schwartz, D. and **Dennis, E.S.** (1986)  
Transposon activity of the *Ac* controlling element in maize is regulated by its degree of  
methylation  
Molecular and General Genetics, 205, 476-482
63. Ellis, J.G., Llewellyn, D.J., **Dennis, E.S.** and Peacock, W.J. (1987)  
Maize *Adh-1* promoter sequences control anaerobic regulation: addition of  
upstream promoter elements from constitutive genes is necessary for  
expression in tobacco  
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64. Llewellyn, D.J., Finnegan, E.J., Ellis, J.G., **Dennis, E.S.** and Peacock, W.J. (1987)  
Structure and expression of an alcohol dehydrogenase 1 gene from *Pisum sativum* (cv.  
"Greenfeast")  
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65. **Dennis, E.S.**, Walker, J.C., Ellis, J.G., Llewellyn, D.J., Singh, K., Peacock, W.J. and Wolstenholme, D. (1987)  
Anaerobic regulation of the maize alcohol dehydrogenase gene  
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66. Ellis, J.G., Llewellyn, D.J., Walker, J.C., **Dennis, E.S.** and Peacock, W.J. (1987)  
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EMBO Journal, 6, 3203-3208
67. Walker, J.C., Howard, E.A., **Dennis, E.S.** and Peacock, W.J. (1987)  
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Proceedings of the National Academy of Sciences, USA, 84, 6624-6628
68. **Dennis, E.S.**, Brettell, R.I.S. and Peacock, W.J. (1987)  
A tissue culture induced *Adh1* null mutant of maize results from a single base change  
Molecular and General Genetics, 210, 181-183
69. Peacock, W.J., Wolstenholme, D., Walker, J.C., Singh, K., Llewellyn, D.J., Ellis, J.G. and **Dennis, E.S.** (1987)  
Developmental and environmental regulation of the maize alcohol dehydrogenase 1 (*Adh1*) gene: Promoter-enhancer interactions  
In "Plant Gene Systems and Their Biology", 263-277  
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# MOLECULAR BIOLOGY OF THE CELL

## SECOND EDITION

Bruce Alberts • Dennis Bray  
Julian Lewis • Martin Raff • Keith Roberts  
James D. Watson



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*Bruce Alberts* received his Ph.D. from Harvard University and is currently Chairman of the Department of Biophysics and Biochemistry at the University of California Medical School in San Francisco. *Dennis Bray* received his Ph.D. from the Massachusetts Institute of Technology and is currently a Senior Scientist in the Medical Research Council Cell Biophysics Unit at King's College London. *Julian Lewis* received his D.Phil. from Oxford University and is currently a Senior Scientist in the Imperial Cancer Research Fund Developmental Biology Unit, Dept. of Zoology, Oxford University. *Martin Raff* received his M.D. degree from McGill University and is currently a Professor in the Biology Department at University College London. *Keith Roberts* received his Ph.D. from Cambridge University and is currently Head of the Department of Cell Biology at the John Innes Institute, Norwich. *James D. Watson* received his Ph.D. from Indiana University and is currently Director of the Cold Spring Harbor Laboratory. He is the author of *Molecular Biology of the Gene* and, with Francis Crick and Maurice Wilkins, won the Nobel Prize in Medicine and Physiology in 1962.

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as transfer RNAs (tRNAs, see p. 103) or form the RNA components of ribosomes (rRNA, see p. 104) or smaller ribonucleoprotein particles.

The amount of RNA made from a particular region of DNA is controlled by *gene regulatory proteins* that bind to specific sites on DNA close to the coding sequences of a gene (see p. 557). In any cell at any given time, some genes are used to make RNA in very large quantities, while other genes are not transcribed at all. For an active gene, thousands of RNA transcripts can be made from the same DNA segment in each cell generation. Because each mRNA molecule can be translated into many thousands of copies of a polypeptide chain, the information contained in a small region of DNA can direct the synthesis of millions of copies of a specific protein. The protein *fibroin*, for example, is the major component of silk. In each silk gland cell, a single fibroin gene makes  $10^4$  copies of mRNA, each of which directs the synthesis of  $10^5$  molecules of fibroin—producing a total of  $10^9$  molecules of fibroin in just 4 days.

### Eucaryotic RNA Molecules Are Spliced to Remove Intron Sequences<sup>15</sup>

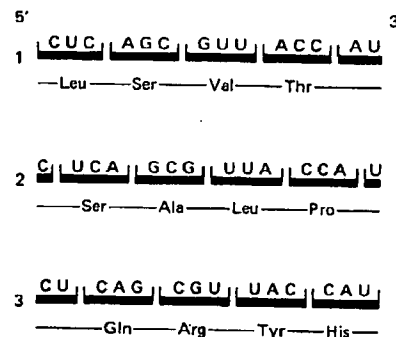
In bacterial cells most proteins are encoded by a single uninterrupted stretch of DNA sequence that is copied without alteration to produce an mRNA molecule. In 1977 molecular biologists were astonished by the discovery that most eucaryotic genes have their coding sequences (called *exons*) interrupted by noncoding sequences (called *introns*). To produce a protein, the entire length of the gene, including both its introns and its exons, is first transcribed into a very large RNA molecule—the *primary transcript*. Before this RNA molecule leaves the nucleus, a complex of RNA processing enzymes removes all of the intron sequences, thereby producing a much shorter RNA molecule. After this *RNA processing* step, called *RNA splicing*, has been completed, the RNA molecule moves to the cytoplasm as an mRNA molecule that directs the synthesis of a particular protein (see Figure 3-13).

This seemingly wasteful mode of information transfer in eucaryotes is presumed to have evolved because it makes protein synthesis much more versatile. For example, the primary RNA transcripts of some genes can be spliced in various ways to produce different mRNAs, depending on the cell type or stage of development. This allows different proteins to be produced from the same gene. Moreover, because the presence of numerous introns facilitates genetic recombination events between exons, this type of gene arrangement is likely to have been profoundly important in the early evolutionary history of genes—speeding up the process whereby organisms evolve new proteins from parts of preexisting ones instead of evolving totally new sequences (see p. 602).

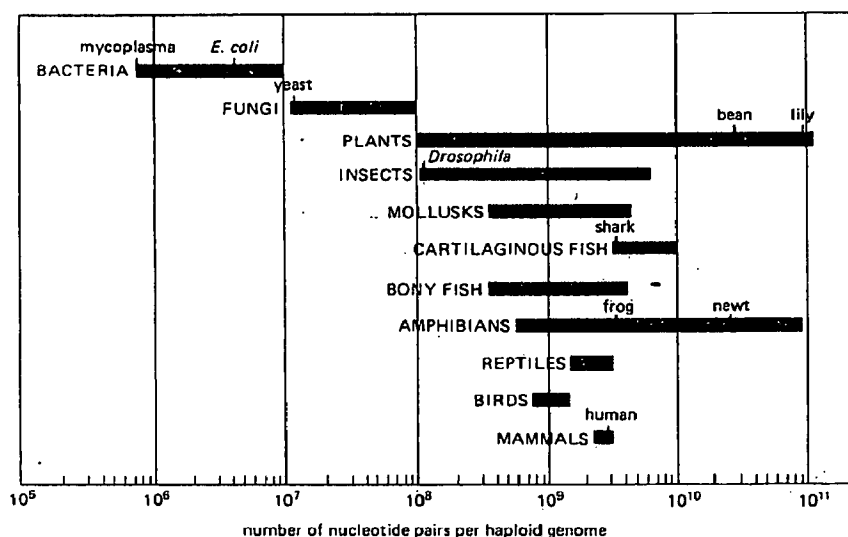
### Sequences of Nucleotides in mRNA Are “Read” in Sets of Three and Translated into Amino Acids<sup>16</sup>

The rules by which the nucleotide sequence of a gene is translated into the amino acid sequence of a protein, the so-called *genetic code*, were deciphered in the early 1960s. The sequence of nucleotides in the mRNA molecule that acts as an intermediate was found to be read in serial order in groups of three. Each triplet of nucleotides, called a *codon*, specifies one amino acid. In principle, each RNA sequence can be translated in any one of three different *reading frames* depending on where the decoding process begins (Figure 3-14). In almost every case, only one of these reading frames will produce a functional protein. Since there are no punctuation signals except at the beginning and end of the RNA message, the reading frame is set at the initiation of the translation process and is maintained thereafter.

Since RNA is a linear polymer of four different nucleotides, there are  $4^3 = 64$  possible codon triplets (remember that it is the *sequence* of nucleotides in the triplet that is important). However, only 20 different amino acids are commonly found in proteins, so that most amino acids are specified by several codons; that is, the genetic code is *degenerate*. The code (shown in Figure 3-15) has been highly conserved during evolution: with a few minor exceptions, it is the same in organisms as diverse as bacteria, plants, and humans.



**Figure 3-14** The three possible reading frames in protein synthesis. In the process of translating a nucleotide sequence into an amino acid sequence, the sequence of nucleotides in an mRNA molecule is read from the 5' to the 3' end in sequential sets of three nucleotides. In principle, therefore, the same RNA sequence can specify three completely different amino acid sequences, depending on the “reading frame.”



**Figure 9-6** The amount of DNA in a haploid genome varies over a 100,000-fold range from the smallest procaryotic cell, the mycoplasma, to the large cells of some plants and amphibia. Note that the genome size of humans ( $3 \times 10^9$  nucleotide pairs) is much smaller than that of some other organisms.

about 10 times more complex than the fruit fly *Drosophila*, which is estimated to have about 5000 essential genes (see p. 510).

Whatever the nonessential DNA in higher eucaryotic chromosomes may do (see Chapter 10, p. 607), the data shown in Figure 9-6 make it clear that it is not a great handicap for a higher eucaryotic cell to carry a large amount of extra DNA. Indeed, even the essential coding regions are often interrupted by long stretches of noncoding DNA.

### Each Gene Is a Complex Functional Unit for the Regulated Production of an RNA Molecule

The primary function of the genome is to produce RNA molecules. Selected portions of the DNA nucleotide sequence are copied into a corresponding RNA nucleotide sequence, which either (as mRNA) encodes a protein or forms a "structural" RNA, such as a tRNA or rRNA molecule. Each region of the DNA helix that produces a functional RNA molecule constitutes a **gene**.

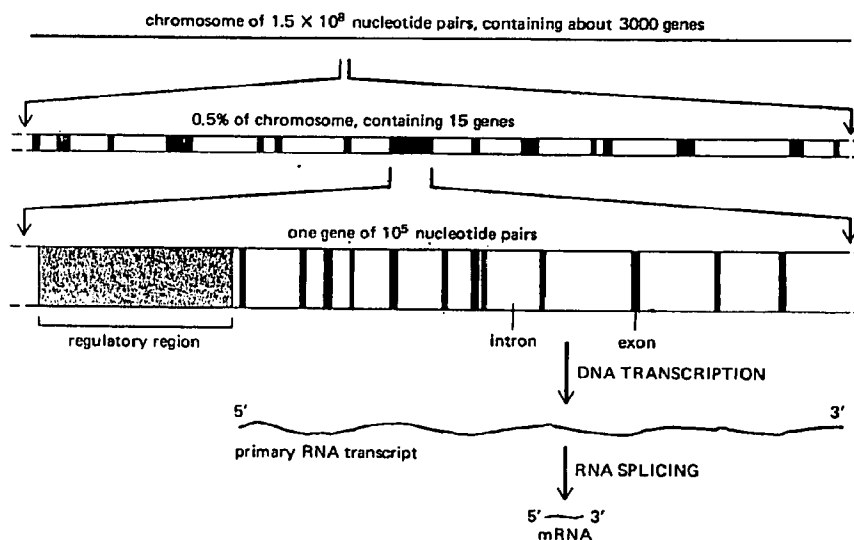
Genes in a chromosome of a higher eucaryote can contain as many as 2 million DNA nucleotide pairs, and genes more than 100,000 nucleotide pairs in length are common (Table 9-1); yet only about 1000 nucleotide pairs are required to encode

**Table 9-1** The Size of Some Human Genes in Thousands of Nucleotides

	Gene Size	mRNA Size	Number of Introns
$\beta$ -Globin	1.5	0.6	2
Insulin	1.7	0.4	2
Protein kinase C	11	1.4	7
Albumin	25	2.1	14
Catalase	34	1.6	12
LDL receptor	45	5.5	17
Factor VIII	186	9	25
Thyroglobulin	300	8.7	36
Dystrophin*	more than 2000	17	more than 50

The size specified here for a gene includes both its transcribed portion and nearby regulatory DNA sequences. (Compiled from data supplied by Victor McKusick.)

\*An altered form of this gene causes Duchenne muscular dystrophy.



**Figure 9-7** The organization of genes on a typical vertebrate chromosome. Proteins that bind to the DNA in regulatory regions determine whether a gene is transcribed; although often located on the 5' side of a gene, as shown here, regulatory regions can also be located in introns, in exons, or on the 3' side of a gene. The intron sequences are removed from the primary RNA transcripts that encode protein molecules to produce a messenger RNA (mRNA) molecule. The figure given here for the number of genes per chromosome is only a minimal estimate.

a protein of average size (one containing 300 to 400 amino acid residues). Most of the extra length consists of long stretches of noncoding DNA that interrupt the relatively short segments of coding DNA. The coding sequences are called **exons**, the intervening (noncoding) sequences are called **introns**. The RNA molecule (called a *primary RNA transcript*) synthesized from such a gene is altered to remove the intron sequences during its conversion to an mRNA molecule (see Figure 9-2) in the process of *RNA splicing* (see p. 531).

Large genes consist of a long string of alternating exons and introns, with most of the gene consisting of introns. In addition, each gene contains *regulatory DNA sequences*, which bind *gene regulatory proteins* that control transcription of the gene. Many regulatory sequences are located "upstream" (on the 5' side) of the site where the RNA transcript begins, but they can also be located in introns, "downstream" (on the 3' side) of the site where the RNA transcript ends, or even in exons. A typical vertebrate chromosome is illustrated schematically in Figure 9-7, along with one of its many genes.

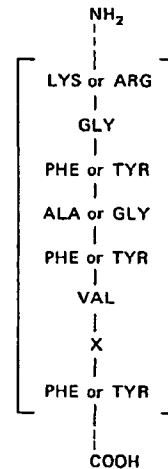
#### Comparisons Between the DNAs of Related Organisms Distinguish Conserved and Nonconserved Regions of DNA Sequence<sup>5</sup>

Technical improvements in DNA sequencing are expected to allow the routine sequencing of stretches of chromosomal DNA that are millions of nucleotide pairs long, so that one can foresee the eventual determination of the sequence of all  $3 \times 10^9$  nucleotides of the human genome. If more than 90% of this sequence is unimportant, however, it will be crucial to have some way of identifying the small proportion of sequence that is important. One way to achieve this is by the simultaneous sequencing of the corresponding regions of a related genome, such as that of the mouse. Human beings and mice are thought to have diverged from a common mammalian ancestor about  $80 \times 10^6$  years ago, which is long enough for roughly two out of every three nucleotides to have been changed by random mutational events (see p. 220). Consequently, the only regions that will have remained closely similar (*conserved* regions) in the two genomes are those where mutations would impair function. (The organisms with these deleterious mutations would have been eliminated from the population by natural selection—see p. 221.) Thus, in general, *nonconserved* regions represent noncoding DNA—both between genes and in introns—whose DNA sequence is not critical for function. Conserved regions, in contrast, represent functionally important exons and regulatory regions. By revealing in this way the results of a very long natural "experiment," comparative DNA sequencing studies highlight the most interesting re-





**Figure 9-78** An amino acid sequence found in many eucaryotic RNA-binding proteins. This consensus—found in proteins from organisms as diverse as yeasts, *Drosophila*, and humans—is present in the proteins of hnRNP particles, in the protein bound to the poly-A tail of hnRNAs, in several snRNP proteins, and in the abundant nucleolar protein, nucleolin. When this sequence is found in a protein of unknown function, it suggests that the protein binds to RNA.



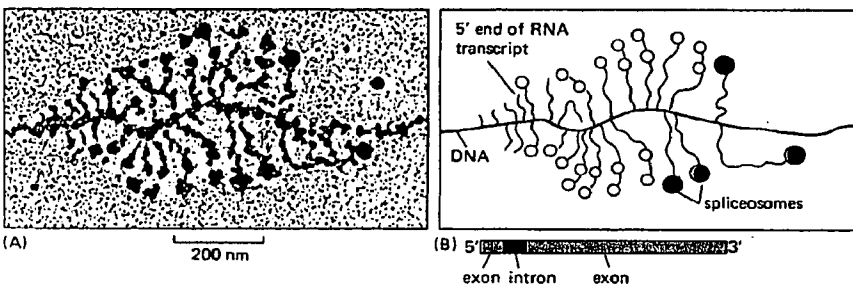
Individual snRNPs are believed to recognize specific nucleic acid sequences through RNA-RNA base-pair complementarity. Some mediate RNA splicing, some are involved in the cleavage reactions that generate the 3' ends of some newly formed RNAs (see p. 596), while the function of others is unknown. The evidence for the role of snRNPs in RNA splicing comes from experiments on RNA processing *in vitro*.

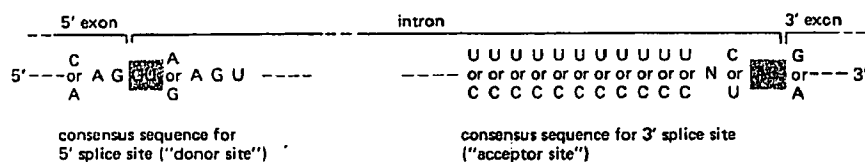
### Intron Sequences Are Removed as Lariat-shaped RNA Molecules<sup>50</sup>

**Introns** range in size from about 80 nucleotides to 10,000 nucleotides or more. They differ dramatically from exons in that their exact nucleotide sequences seem to be unimportant. Thus introns have accumulated mutations rapidly during evolution, and it is often possible to alter most of the nucleotide sequence of an intron without greatly affecting gene function. This has led to the suggestion that intron sequences have no function at all and are largely genetic "junk," a proposition we shall examine later (see p. 602). The only highly conserved sequences in introns are those required for intron removal. Thus there are consensus sequences at each end of an intron that are nearly the same in all known intron sequences, and these cannot be altered without affecting the splicing process that normally removes the intron sequence from the primary RNA transcript. These conserved boundary sequences at the 5' splice site (**donor site**) and the 3' splice site (**acceptor site**) are shown in Figure 9-80. The RNA breaking and rejoining reactions must be carried out precisely because an error of even one nucleotide would shift the reading frame in the resulting mRNA molecule and make nonsense of its message.

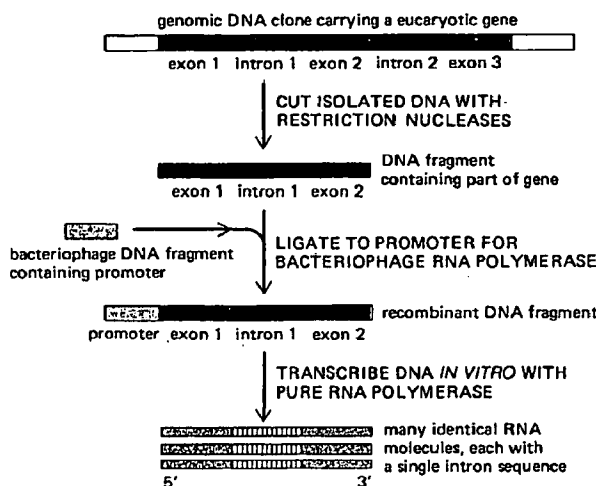
The pathway by which the intron sequences are removed from primary RNA transcripts has been elucidated by *in vitro* studies in which a pure RNA species containing a single intron is prepared by incubating an appropriately designed DNA fragment with a highly active, purified RNA polymerase (Figure 9-81). When these RNA molecules are added to a cell extract, they become spliced in a two-step enzymatic reaction that requires prolonged incubation with ATP, selected proteins in the extract, and the U1, U2, U5, and U4/U6 snRNPs; these components assemble into a large multicomponent ribonucleoprotein complex, or **spliceosome**. Characterization of the RNA species that appear as intermediates during the reaction, as well as the snRNPs required to produce them, led to the discovery

**Figure 9-79** Electron micrograph of a chromatin spread showing large ribonucleoprotein particles assembling at the 5' and 3' splice sites to form a spliceosome. In the micrograph (A) a gene encoding a *Drosophila* chorion protein has been identified, so that the positions of the splice sites on the primary RNA transcript are known. (B) Most of the RNA transcripts have either one or two large RNP particles near their 5' ends. When there are two particles on a transcript (open circles in (B)), they average 25 nm in diameter and occur at or very near the positions of the 5' and 3' splice sites for the single small intron sequence (228 nucleotides long) near the 5' end of the transcripts. The more mature, longer transcripts on the two genes frequently display a single larger particle (colored circles in (B)) in the region of the intron, which probably results from the stable association of the two smaller particles and represents the assembled spliceosome. Since splicing occurs in some cases while the 3' end of the RNA chain is still being transcribed, the poly A at the 3' end of hnRNA molecules cannot be required for splicing. Most of the major hnRNP proteins have been removed from these transcripts by the spreading conditions used. (Adapted from Y.N. Osheim, O.L. Miller, and A.L. Beyer, *Cell* 43:143-151, 1985.)

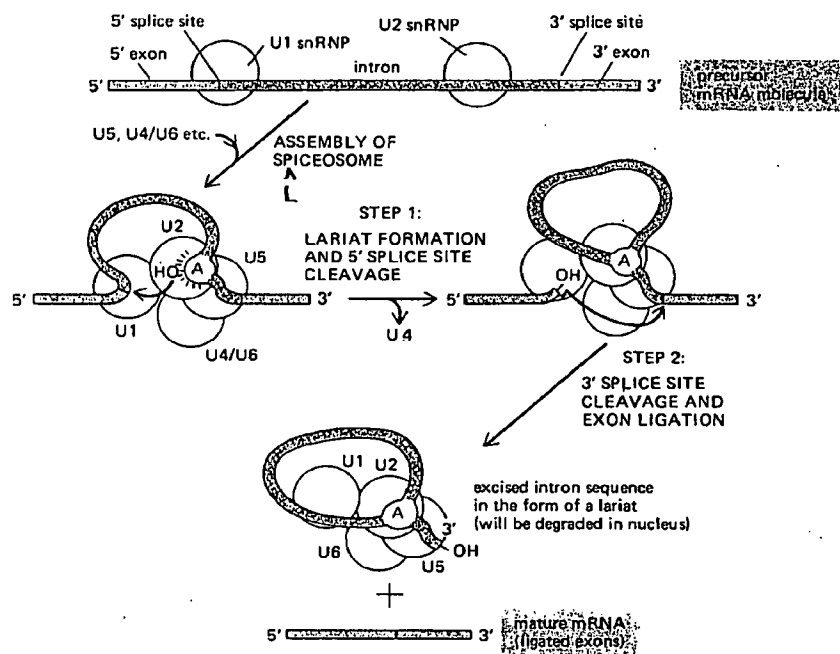




**Figure 9-80** Consensus sequences for the 5' and 3' splice sites used in RNA splicing. The sequence given is that for the RNA chain; the nearly invariant GU and AG dinucleotides at either end of the intron are shaded in color (see also Figure 9-77).



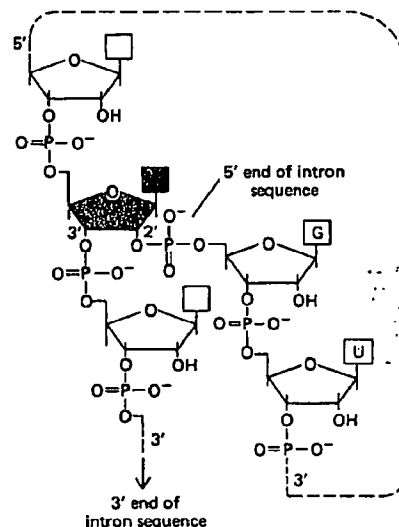
**Figure 9-81** Outline of the procedure used to produce abundant amounts of pure RNA molecules for the analysis of RNA splicing *in vitro*. The method depends on the ability to produce large amounts of any desired DNA sequence by genetic engineering and DNA cloning (see p. 258) as well as on the availability of relatively simple RNA polymerases from bacteriophages T7 or SP6, which transcribe DNA with high efficiency *in vitro*. By coupling a eucaryotic DNA fragment to a bacteriophage promoter, a bacteriophage RNA polymerase can be used to generate *in vitro* large amounts of the RNA encoded by the eucaryotic DNA fragments. The 5' cap present on hnRNAs can be incorporated into such RNAs by using a chemically synthesized, capped nucleotide to initiate the transcription process (not shown).



**Figure 9-82** Catalysis of RNA splicing by a spliceosome formed from the assembly of U1, U2, U5, and U4/U6 snRNPs (shown as circles), plus other components (not shown). After assembly of the spliceosome, the reaction occurs in two steps: in step 1 a special A nucleotide in the intron sequence located close to the 3' splice site attacks the 5' splice site, which is cleaved; the cut 5' end of the intron sequence joins covalently to this A nucleotide, forming the branched nucleotide shown in Figure 9-83. In step 2 the 3'-OH end of the first exon, which was exposed in the first step, adds to the beginning of the second exon, cleaving the RNA molecule at the 3' splice site; the two exon sequences are thereby joined to each other and the intron sequence is released as a lariat. The complete spliceosome complex sediments at 60S, indicating that it is nearly as large as a ribosome. These splicing reactions occur in the nucleus and generate mRNA molecules from primary RNA transcripts (mRNA precursor molecules).

that the intron is excised in the form of a *lariat*, according to the splicing pathway shown in Figures 9-82 and 9-83.

Individual roles have been defined for several of the snRNPs. The U1 snRNP, for example, binds to the 5' splice site, guided by a nucleotide sequence in the U1 RNA that is complementary to the nine-nucleotide splice-site consensus sequence (see Figure 9-80). Since RNA is capable of acting like an enzyme (see p. 105), either the RNA or the protein components of the spliceosome could be responsible for catalyzing the breakage and formation of covalent bonds required for RNA splicing.

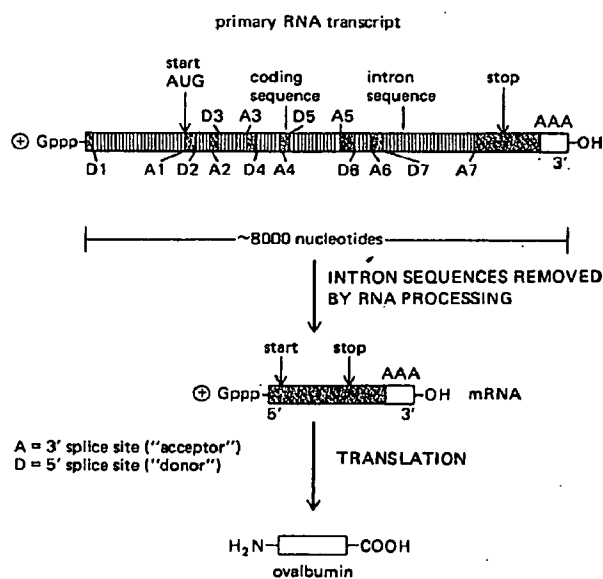


**Figure 9-83** Structure of the branched RNA chain that forms during nuclear RNA splicing. The shaded A is the nucleotide highlighted in Figure 9-82, and the branch is formed in step 1 of the splicing reaction illustrated there. In this step the 5' end of the intron sequence is cleaved and its phosphate group couples covalently to the 2'-OH ribose group of the A nucleotide, which is located about 30 nucleotides from the 3' end of the intron sequence. The branched chain remains in the final excised intron sequence and is responsible for its lariat form (see Figure 9-82).

## Multiple Intron Sequences Are Usually Removed from Each RNA Transcript<sup>51</sup>

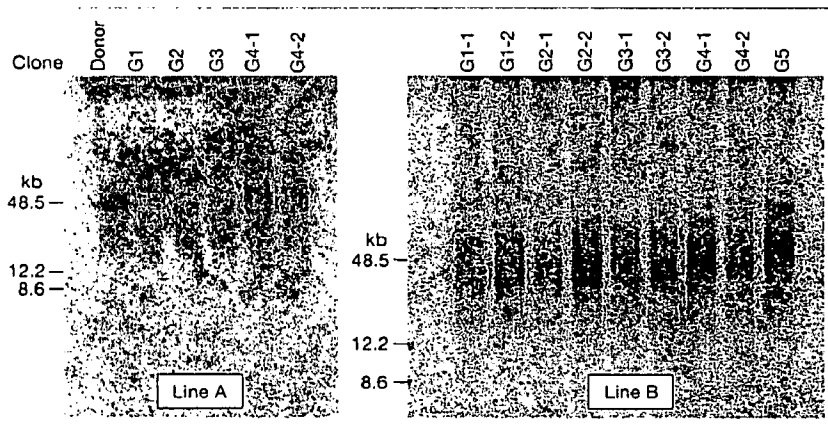
Because the spliceosome seems mainly to recognize a consensus sequence at each intron boundary, the 5' splice site (*donor site*) at the end of any one intron can in principle be joined to the 3' splice site (*acceptor site*) of any other intron in the splicing process. Thus, when the 5' and 3' halves of two different introns are experimentally combined, the resulting hybrid intron sequence is often recognized by the RNA-splicing enzymes and removed.

In view of this result, it is surprising that vertebrate genes can contain as many as 50 introns (see Table 9-1, p. 486). If any two 5' and 3' splice sites were mispaired for splicing, some functional mRNA sequences would be lost, with disastrous consequences. Somehow such mistakes are avoided: the RNA processing machinery normally guarantees that each 5' splice site pairs only with the 3' splice site that is closest to it in the downstream (5'-to-3') direction of the linear RNA sequence (Figure 9-84). How this sequential pairing of splice sites is accomplished is not known, although the assembly of the spliceosome while the RNA transcript is still growing (see Figure 9-79) is presumed to play a major part in ensuring an orderly pairing of the appropriate splice sites. There is also evidence that the exact three-dimensional conformations adopted by the intron and exon sequences in the RNA transcript are important. We shall see, however—both below and in Chapter 10—that splicing can be controlled, and in selected cases the simple pattern of 5'-to-3' splicing does not hold.



**Figure 9-84** The primary RNA transcript for the chicken ovalbumin gene, showing the organized removal of seven introns required to obtain a functional mRNA molecule. The 5' splice sites (donor sites) are denoted by D, and 3' splice sites (acceptor sites) are denoted by A.

## brief communications



**Figure 1** Telomere lengths in successive generations (G1–G5) of mice cloned from cumulus cells. Southern-blot analysis of terminal restriction-enzyme-cut fragments in five sequential generations shows that telomeres do not undergo incremental erosion in successive clonal generations. Genomic DNA isolated from peripheral-blood lymphocytes taken from representative animals from each generation was digested with the restriction enzyme *Hinf*I, resolved on a pulse field gel, transferred to a solid support and probed with a 5'-<sup>32</sup>P-labelled (T<sub>6</sub>AG<sub>3</sub>)<sub>n</sub> oligonucleotide. Peripheral blood lymphocytes were sampled on the same day. Ages of mice (in months) were: in line A, donor, 18; G1, 16; G2, 14; G3, 12; G4, 9; G5, 9; in line B, G1, 15.5; G2, 13; G3, 11; G4, 9; G5, 7. Suffix numbers (G4-1 and G4-2, for example) identify different pups of each generation.

was repeated with cumulus cells from adult G1 mice as nucleus donors to produce the next clonal generation, G2, and so on. Table 1 summarizes the results obtained following the reconstruction of 3,920 enucleated oocytes.

Previously, about 2% of enucleated oocytes receiving a cumulus cell nucleus developed to live-born pups<sup>1</sup>. This value is comparable to the cloning efficiency for G1 in lines A (1.5%) and B (4.2%). However, the success rate dropped in successive cloned generations: line A did not produce a G5 clone from 670 reconstructed oocytes; in line B, the only live-born G6 clone was cannibalized by her foster mother, thereby terminating the line. Mouse lines A and B therefore represent totals of 9 and 26 clones from their respective donors. Placental size was consistently in the range previously reported for cloned mice<sup>2</sup> and did not increase in successive generations (data not shown).

Do sequentially cloned mice show signs of accelerated ageing? We assessed the behaviour of these mice and determined telomere lengths to assess organismal and cellular measures of ageing, respectively. We evaluated learning ability in the Morris water maze and Krushinsky tests, as well as strength and agility, and also used other

assays designed to monitor signs of premature ageing, such as a decline in activity in the home cage and loss of coordination<sup>3</sup>. All cloned mice were, by these criteria, normal compared with age-matched controls (data not shown); the G5 mouse is alive and healthy at 1.5 years.

We measured telomere length in peripheral blood lymphocytes of clones G1–G6 by Southern-blot analysis of terminal restriction-enzyme-digested fragments (Fig. 1) and found no evidence of shortened telomeres in the cloned mice. In fact, our results show that the telomeres lengthen with each generation. As representative animals of each generation were sampled simultaneously, we cannot rule out an age-related contribution to this increase (with younger mice having longer telomeres). In addition, long telomeres in mice are optimally studied by means of different assays such as quantitative fluorescence *in situ* hybridization<sup>4</sup>. We have detected telomerase activity in cumulus cells (data not shown); it is therefore possible that telomeres in these cells are unusually long, resulting in offspring with concomitantly longer telomeres.

Shortened<sup>5</sup> and lengthened<sup>6</sup> telomeres have been reported in cloned livestock but, unlike ours, those experiments involved only a single round of cloning. Our results

on sequentially cloned mice verify that telomere shortening is not a necessary outcome of the cloning process<sup>7</sup>. However, as only 1–2% of reconstructed oocytes yield live-born clones, the possibility of selection for donor nuclei with the longest telomeres cannot be excluded. Further investigation is required into the consequences of nuclear transfer on telomere length and lifespan.

Teruhiko Wakayama\*†, Yoichi Shinkai‡, Kellie L. K. Tamashiro\*, Hiroyuki Niida§, D. Caroline Blanchard||, Robert J. Blanchard||, Atsuo Ogura¶, Kentaro Tanemura¶, Makoto Tachibana‡, Anthony C. F. Perry#, Diana F. Colgan#, Peter Mombaerts#, Ryuzo Yanagimachi\*

\*Department of Anatomy and Reproductive Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822, USA

‡Department of Cell Biology, Institute for Virus Research, Kyoto University, Sakyo-Ku, Kyoto 606-8507, Japan

§Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720, USA

||Beckman Laboratory of Neurobiology, University of Hawaii, Honolulu, Hawaii 96822, USA

¶Department of Veterinary Science, National Institute of Infectious Disease, Tokyo 162-8640, Japan

#The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

†Present address: The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

e-mail: wakayama@mail.rockefeller.edu

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## Gene expression

## Total silencing by intron-spliced hairpin RNAs

Post-transcriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism inherent in many life-forms, can be induced in plants by transforming them with either antisense<sup>1</sup> or co-suppression<sup>2</sup> constructs, but typically this results in only a small proportion of silenced individuals. Here we show that gene constructs encoding intron-spliced RNA with a hairpin structure can induce PTGS with almost 100% efficiency when directed against viruses or endogenous

**Table 1** Effect of sequential cloning on full-term development

Line	G1	G2	G3	G4	G5	G6	Total
A	2/131 (1.5)	1/228 (0.4)	1/263 (0.4)	5/238 (2.1)	0/670 (0)	-	9/1,530 (0.6)
B	4/96 (4.2)	7/351 (2.0)	5/352 (1.4)	6/286 (2.1)	3/581 (0.5)	1/724 (0.1)	26/2,390 (1.1)

Successive generations are represented as G1, G2 and so on for two independent mouse lines, A and B. The number of pups born live after cumulus-cell

nuclear transfer is compared to successfully reconstructed oocytes (pups/oocytes), with the corresponding percentages in parentheses. Significant  $\chi^2$

comparisons were derived for G4 and G5 from line A, G1 and G5, G6 from line B, and G2, G3, G4 versus G6 from line B ( $P < 0.05$ ).

## brief communications

genes. These constructs could prove valuable in reverse genetics, genomics, engineering of metabolic pathways and protection against pathogens.

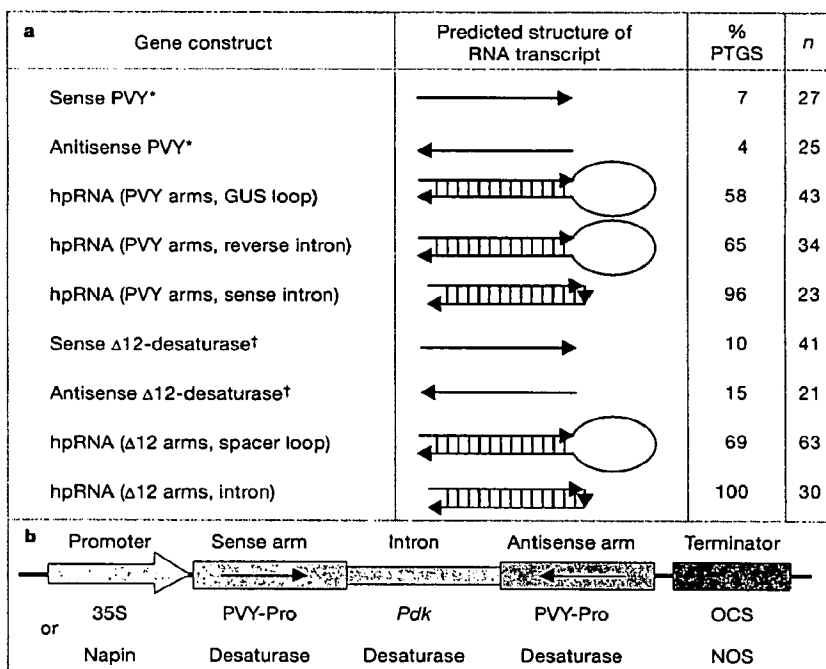
Induction of PTGS by co-suppression and antisense methods that target the *Nia*-protease (*Pro*) gene sequence of potato virus Y (PVY)<sup>3</sup> cause silencing in 7% and 4% of independent transformants, respectively; induction of PTGS in these tobacco plants (*Nicotiana tabacum*) manifests as immunity<sup>4,5</sup> to the virus.

Using principles we developed for silencing constructs that express double-stranded RNA and inverted-repeat RNAs<sup>3</sup>, we made a construct encoding a single self-complementary hairpin RNA (hpRNA) of the *Pro* sequence. The construct contains sense and antisense *Pro* sequences flanking an 800-nucleotide spacer fragment derived from the *uidA* (GUS) gene (Fig. 1a). About 60% (25/43) of the plants that are transformed with this construct, many of which contained a single transgene copy, were immune to the virus. The spacer fragment contributed to the stability of the perfect inverted-repeat sequences, but it was not required for the specificity of the PTGS (Fig. 1a).

To test the effect of removing the loop region of hpRNA, we replaced the spacer with an intron sequence (Fig. 1a, b). The intron sequence provides stability to the DNA, but is spliced out during pre-mRNA processing<sup>6</sup> to produce loopless hpRNA. As a control, we made a sister construct in which the intron sequence was inserted in the reverse, non-splicing, orientation. When transformed into tobacco, 22 of 34 (65%) reverse-intron plants were immune, a similar frequency to plants transformed with the GUS spacer construct. Amazingly, we found that 22 of 23 plants transformed with the construct containing the functional intron were immune to the virus.

To test whether this enhancement by the sense-intron construct was a general phenomenon, we made two hpRNA constructs against the endogenous  $\Delta 12$ -desaturase (*Fad2*) gene of *Arabidopsis*, which catalyses the conversion of oleic to linoleic acid in the seed<sup>6,7</sup>; one construct contained an intron and the other a non-intron spacer region. We found that 69% (44/63) of the transgenic plants with the non-intron spacer region construct showed PTGS of the  $\Delta 12$ -desaturase gene, but that 100% (30/30) of plants transformed with the intron construct showed silencing of this gene.

How does the presence of this intron enhance silencing efficiency? The process of intron excision from the construct by the spliceosome might help to align the complementary arms of the hairpin in an environment favouring RNA hybridization, promoting the formation of a duplex. Alternatively, splicing may transiently increase



**Figure 1** Efficiency of induction of post-transcriptional gene silencing (PTGS) by different gene constructs and the predicted structure of RNA transcribed from the transgenes. **a**, PTGS efficiency measured for potato virus (PVY) and  $\Delta 12$ -desaturase genes as the percentage of independent transgenic plants immune to PVY and the percentage of plants with enzyme activity reduced by more than 20% compared with wild type, respectively. In the predicted structures of RNA transcripts, right- and left-pointing arrows represent sense and antisense orientation of sequences, respectively; small vertical arrows represent splice-junction sequences remaining after the intron has been spliced out. Vertical lines in the predicted structures indicate duplex formation. Asterisks, data from ref. 3; daggers, data from ref. 7; hpRNA, hairpin RNA; n, number of independent transformants; GUS,  $\beta$ -glucuronidase. **b**, Design of intron-containing hairpin constructs. OCS, octopine synthase; NOS, nopaline synthase.

the amount of hairpin RNA by facilitating, or retarding, the hairpin's passage from the nucleus, or by creating a smaller, less nuclease-sensitive loop.

Our PVY constructs contained intron-2 from the *Pdk* gene of *Flaveria*<sup>8</sup>, whereas the  $\Delta 12$ -desaturase construct contained intron-1 from the *Arabidopsis Fad2* gene (Fig. 1b). PVY constructs were controlled by the constitutive CaMV35S (ref. 9) promoter and produced hpRNA containing the PVY coding-region sequence (700 nucleotides); the desaturase gene construct used the seed-specific napin promoter<sup>10</sup> to produce hpRNA representing 120 nucleotides of the 3'-untranslated region of the  $\Delta 12$ -desaturase gene.

We believe that constructs encoding intron-hpRNA should efficiently induce PTGS for a wide range of genes in a variety of circumstances and could become as useful to plant biology as RNAi<sup>11,12</sup> is to the study of nematodes and *Drosophila*. The transgene design might also have applica-

tion in organisms in which RNAi has been obtained by injection of double-stranded RNA.

Neil A. Smith, Surinder P. Singh, Ming-Bo Wang, Peter A. Stoutjesdijk, Allan G. Green, Peter M. Waterhouse  
CSIRO Plant Industry, Canberra, ACT 2601, Australia

e-mail: peter.waterhouse@pi.csiro.au

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## TECHNICAL ADVANCE

# Construct design for efficient, effective and high-throughput gene silencing in plants

S. Varsha Wesley<sup>1,†</sup>, Christopher A. Helliwell<sup>1,†</sup>, Neil A. Smith<sup>1,†</sup>, MingBo Wang<sup>1</sup>, Dean T. Rouse<sup>1</sup>, Qing Liu<sup>1</sup>, Paul S. Gooding<sup>1</sup>, Surinder P. Singh<sup>1</sup>, David Abbott<sup>1</sup>, Peter A. Stoutjesdijk<sup>1</sup>, Simon P. Robinson<sup>1</sup>, Andrew P. Gleave<sup>2</sup>, Allan G. Green<sup>1</sup> and Peter M. Waterhouse<sup>1,\*</sup>

<sup>1</sup>CSIRO Plant Industry, PO Box 1600 Canberra ACT 2601, Australia, and

<sup>2</sup>HortResearch, Mt Albert Research Centre, Private Bag 92 169, Auckland, New Zealand

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\*For correspondence (fax +61 262465000; e-mail peterw@pi.csiro.au).

<sup>†</sup>These authors contributed equally to this work.

## Summary

Post-transcriptional silencing of plant genes using anti-sense or co-suppression constructs usually results in only a modest proportion of silenced individuals. Recent work has demonstrated the potential for constructs encoding self-complementary 'hairpin' RNA (hpRNA) to efficiently silence genes. In this study we examine design rules for efficient gene silencing, in terms of both the proportion of independent transgenic plants showing silencing, and the degree of silencing. Using hpRNA constructs containing sense/anti-sense arms ranging from 98 to 853 nt gave efficient silencing in a wide range of plant species, and inclusion of an intron in these constructs had a consistently enhancing effect. Intron-containing constructs (ihpRNA) generally gave 90–100% of independent transgenic plants showing silencing. The degree of silencing with these constructs was much greater than that obtained using either co-suppression or anti-sense constructs. We have made a generic vector, pHANNIBAL, that allows a simple, single PCR product from a gene of interest to be easily converted into a highly effective ihpRNA silencing construct. We have also created a high-throughput vector, pHELLSGATE, that should facilitate the cloning of gene libraries or large numbers of defined genes, such as those in EST collections, using an *in vitro* recombinase system. This system may facilitate the large-scale determination and discovery of plant gene functions in the same way as RNAi is being used to examine gene function in *Caenorhabditis elegans*.

**Keywords:** PTGS, RNAi, genomics, vector, ihpRNA, Gateway.

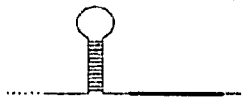
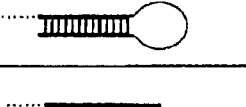
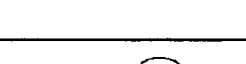
## Introduction

The ultimate goal of current genome projects is to identify the biological function of every gene in the genome. Whole genomes of several organisms (including *Arabidopsis*, <http://www.arabidopsis.org>), have been completely sequenced, providing a wealth of information. The functions of some of the genes have been identified directly by the appropriate assay, or have been inferred by homology to genes of known function in other organisms. Loss-of-function mutants, from insertional mutagenesis or transposable elements, have also been very informative about the role of some of

these genes (AzpirozLeehan and Feldmann, 1997; Martienssen, 1998), particularly in the large-scale analysis of the yeast genome (Ross-Macdonald *et al.*, 1999). However, the functions of a large proportion of genes remain unknown.

Injection or ingestion of dsRNA into nematodes can trigger specific RNA degradation, in a process known as RNA-interference (RNAi; Fire *et al.*, 1998). This process facilitates targeted post-transcriptional gene silencing (PTGS) and has recently been harnessed to study the function of over 4000 genes on chromosomes I and III

(a)

Construct	Predicted RNA structure	PTGS	<i>n</i>
Adj-hp RNA		25 %	8
hpRNA		85 %	14
sRNA		30 %	10

(b)


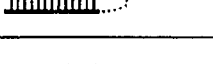
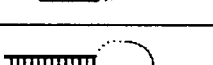
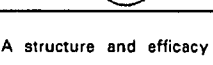
hpRNA		55 %	234
ihpRNA		90 %	243
ihpRNA overhang		80 %	48
ihpRNA spacer		89 %	36

Figure 1. The predicted RNA structure and efficacy of gene-silencing constructs.

(a) Three constructs, controlled by Ubi1 promoter, silencing GUS in rice. Thick lines indicate a 580 nt GUS sequence; grey lines indicate non-GUS sequences; dashed grey lines indicate intron-junction sequences left after splicing; and short lines within the stem of hairpin structures indicate base pairing. Numbers in PTGS column indicate the percentage of plants showing GUS silencing; *n* = number of plants in each treatment.

(b) Silencing efficacy of four different construct types with sequences as depicted in (a), except the thick lines in hpRNA and ihpRNA represent the various different target sequences in Table 1; and the thick lines in iphRNAoverhang and iphRNAspacer represent PVY Pro sequences. The percentage PTGS of hpRNA and ihpRNA, and iphRNAoverhang and iphRNA spacer, are the average percentage silencing of these types of constructs reported in Table 1 and the percentage of plants showing immunity to PVY, respectively.

in *Caenorhabditis elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). We discovered that transgenes designed to express double-stranded or single-stranded self-complementary (hairpin) RNA have a similar post-transcriptional silencing effect in plants (Wang and Waterhouse, 2000; Waterhouse *et al.*, 1998) and that, in at least two examples, almost 100% of plants transformed with an intron-containing hairpin RNA construct showed silencing (Smith *et al.*, 2000). These results led us to ask whether hpRNA technology might be exploited for gene discovery in plants. We have sought to design and evaluate generic intron-hpRNA constructs that might enable plant gene-discovery studies on a scale that matches those in nematodes.

## Results

### Location of silence-inducing sequences in hairpin RNA constructs

Constructs encoding RNAs with regions of self-complementarity efficiently induce gene silencing. We have previously shown that the sequences in the duplex stem in hpRNAs direct gene silencing (Smith *et al.*, 2000; Waterhouse *et al.*, 1998), whereas the results of Hamilton *et al.* (1998) suggest that single-stranded RNA sequences adjacent to a potential hairpin-forming structure give sequence specificity to silencing. The latter arrangement (adj-hpRNA) could be easily incorporated into gene-silencing vectors as the sequence encoding the hairpin RNA could be generic to the vector, while the specificity of the silencing would be accomplished by simply inserting a single copy of target gene sequence. In contrast, hpRNA constructs require two copies of the target sequence in an inverted-repeat orientation, in order to produce duplex RNA. To compare the relative efficacy of the designs, various GUS-silencing constructs, under the control of the Ubi1 promoter and associated intron, were made (Figure 1a) and super-transformed into GUS-expressing rice. Histochemical staining of the transformed plants showed that the adj-hp RNA construct gave no higher frequency of silenced lines than conventional co-suppression (sRNA), but the hpRNA construct gave many more silenced lines (Figure 1a). This suggested that the hpRNA was the design of choice.

Examination of the stained rice Ubi-hpGUS plants and a similar 35S-hpGUS construct in tobacco (Figure 2f) showed that the silencing was evenly distributed throughout the plant. Analysis of RNAs in the tobacco plants for the presence of GUS-derived small ( $\approx 21$  nt) RNAs showed a perfect correlation between the presence of these molecules and the presence of the 35S-hpGUS construct and silencing of the target GUS gene (Figure 3). This confirms that the silencing was due to PTGS; such small RNAs are a hallmark of PTGS (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001).

### Intron-spliced hpRNA vectors

We have found that it is necessary to include a spacer region between the arms of hpRNA constructs for stability of the inverted repeat DNA in *Escherichia coli*. However, replacing the spacer (loop) region of hpRNA constructs with a functional intron sequence increases the proportion of independent silenced lines recovered from approximately 50 to about 100% (Smith *et al.*, 2000). In these experiments, the targets were potato virus Y (PVY) and the FAD2  $\Delta 12$ -desaturase gene of *Arabidopsis*. The constructs were designed such that their pre-mRNA should splice to

form hpRNAs with small loops (Figure 4). The PVY construct should give an hpRNA comprising a 730 nt stem and a 6 nt loop; the hpRNA from the  $\Delta 12$ -desaturase construct should contain a 120 nt stem and a 21 nt loop. Although the PVY construct contained only two and four bases of original exon sequence 5' and 3' of the intron, respectively, the intron was still functional. RT-PCR and sequencing of transgene mRNA in plants containing the *HindIII* intron fragment (Figure 4) showed that the intron was cleaved out, leaving the predicted splice junction (data not shown).

The  $\Delta 12$ -desaturase result (100% of independent plants showing silencing despite having a 21 nt loop in the hpRNA) showed that the intron-enhanced silencing was not solely due to the tightness of the hairpin loop. Therefore we wondered whether this could be exploited to make a generic intron-spliced hpRNA (ihpRNA) vector into which the gene, or gene fragment, of choice could be directionally cloned to make sense and anti-sense arms. The vector pHANNIBAL (Figures 4 and 5), and a sister vector, pKANNIBAL (with bacterial ampicillin and kanamycin resistance genes, respectively), were designed so that a PCR fragment could be inserted in the sense orientation into the *XhoI*.*EcoRI*.*KpnI* polylinker and in the anti-sense orientation in the *Clal*.*HindIII*.*BamHI*.*XbaI* polylinker. This may be accomplished either by two separate PCR reactions with the appropriate single sites introduced with each primer, or by a single PCR using primers each introducing two restriction sites (e.g. primer 1, *XbaI*.*XhoI*.xxx; primer 2, *Clal*.*KpnI*.xxx). The construct will produce an hpRNA with a loop of 30–50 bases depending on which restriction sites are used to insert the targeting gene sequences.

The efficacy of pHANNIBAL was tested in *Arabidopsis* targeting the pigment biosynthesis gene chalcone synthase (*CHS*); the ethylene signalling gene *EIN2*; and the flowering repression gene *FLC1*. These genes were chosen because their mutant alleles have been reported in *Arabidopsis* to give distinct phenotypes. The *tt4* (CS85) EMS mutant (Koornneef *et al.*, 1990) produces inactive CHS, resulting in reduced production of flavonoid pigments in both the stem and seed coat. The mutant *ein2* (Alonso *et al.*, 1999) is insensitive to ethylene and grows well on media containing 1-aminocyclopropane-1-carboxylic acid, whereas wild-type plants develop a very stunted appearance when grown on such media. The mutant *flc1* (Amasino *et al.*, 2000) flowers earlier than wild-type *Arabidopsis*.

A 741 nt piece of CHS coding region was amplified from *A. thaliana* (*Landsberg erecta*) using primers that added an *XhoI* and a *KpnI* site on the ends of one product and an *XbaI* and *BamHI* site on the ends of the other product. These two amplification products were then directionally cloned into pHANNIBAL (Figure 5).

Similar cloning strategies were adopted for a 600 nt sequence from *EIN2*, and both a 650 nt and a 400 nt sequence from *FLC1*. As controls, sense and anti-sense constructs of CHS and an anti-sense construct of *FLC1* were also generated. All the constructs were subcloned into the binary vector pART27 and transformed into *Arabidopsis*.

Only two of the 19 plants transformed with the CHS co-suppression construct, and none of the 25 plants transformed with the CHS anti-sense construct, showed any obvious evidence of silencing. Whereas over 90% (21 of 23) of the plants transformed with the CHS-HANNIBAL constructs showed pronounced silencing (Table 1). The seed colours of most of these lines were virtually indistinguishable, to the naked eye, from seed of the *tt4*(CS85) mutant (Figure 2a). Examination of the seed under a light microscope revealed that the degree of pigmentation was generally uniform in the cells of the coat of an individual seed, and among seeds of the same line (Figure 2b,c). There was a perceptible difference in the levels of pigmentation between the different lines. The relative flavonoid content of seed from three lines selected to span the range of seed colour in the plant lines transformed with CHS-HANNIBAL, and from the co-suppression line giving the lightest coloured seed were 7, 23, 47 and 75%, respectively. The *tt4* (CS85) and wild-type seed had values of 0 and 100%, respectively.

Sixty-four independent lines transformed with the EIN-HANNIBAL construct were obtained. The progeny from 42 of the lines showed Mendelian segregation for normal and stunted growth when grown on ACC medium, whereas all wild-type plants showed a stunted morphology on this medium (Table 1; Figure 2e).

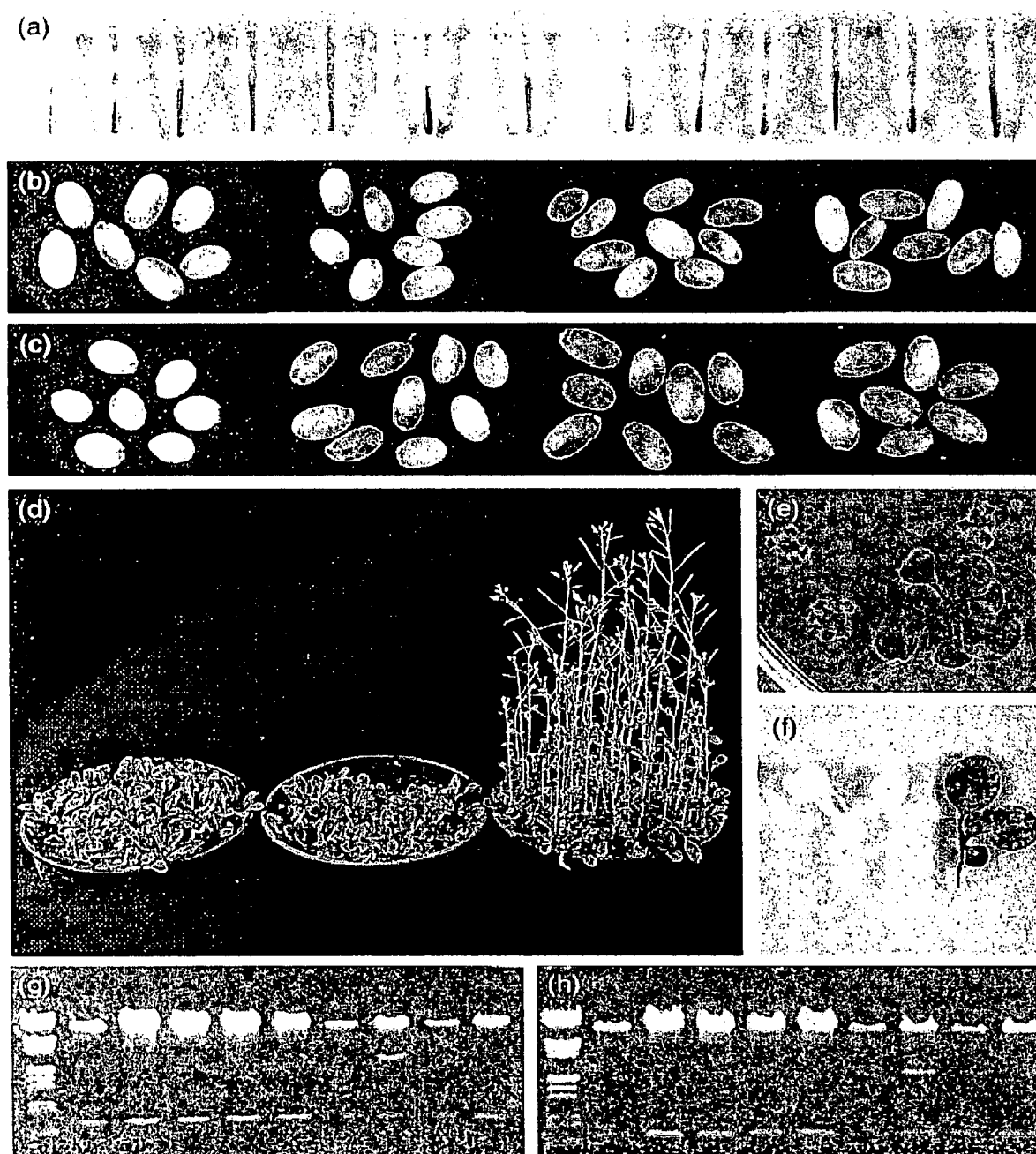
The transgenic progeny of every one of the 31 independent plants transformed with the *FLC1*-HANNIBAL constructs flowered earlier and made fewer leaves, prior to flowering, than wild-type plants (Table 1; Figures 2d and 6). The transgenic progeny from the majority of the plants transformed with the *FLC1* anti-sense construct had similar leaf numbers and flowering times to those of wild-type plants. The flowering time and leaf number provided an easy measurement of the degree of silencing in individual lines. Only two of the 31 *FLC1*-HANNIBAL plant lines did not flower in less than 25 days after germination, whereas only two anti-sense plant lines flowered in less than 30 days. This suggests that ihpRNA constructs not only give an increased proportion of silenced transformants than anti-sense constructs, but also give more profound levels of silencing. However, even the most profoundly silenced *FLC1*-HANNIBAL line flowered 1 day later than the *flc* mutant, suggesting that it was not quite the equivalent of a null allele.

Collectively, pHANNIBAL-based constructs (which are driven by the constitutive 35S promoter) have been



made for five different genes (*CHS*, *EIN2*, *FLC1*, PVY-Pro and polyphenol oxidase – *PPO*). Similar intron-containing constructs targeting seed specifically against two different genes ( $\Delta 12$ - and  $\Delta 9$ -desaturase) in *Arabidopsis* and/or cotton have been tested for their silencing efficiency. Intron-less hpRNA, anti-sense and co-suppres-

sion constructs have also been used in many of these gene/host combinations. The results are summarized in Table 1. The ihpRNA constructs were effective, with arm lengths ranging from 98 to 853 nt, giving 66–100% (average 90%) independent silenced transformants. Intron-free hpRNA constructs gave 48–69% (average 58%) silenced



transformants, and conventional co-suppression or anti-sense constructs gave 0–30% (average 13 and 12%, respectively) silenced transformants. The intron-spliced or intron-free hpRNA constructs were effective when targeted against the coding, 5' untranslated or 3' untranslated regions of the mRNA. Taken together, these results indicate that ihpRNA constructs consistently give the most efficient silencing under a wide range of conditions.

#### Effect of intron location and unbalanced arms in pHANNIBAL

Intron-spliced hpRNA constructs appear to give a higher proportion of silenced transformants than intron-free hpRNA constructs. One explanation for this might be that the process of intron-splicing aligns the arms of the hpRNA, facilitating their duplex formation in the spliceosome complex, whereas the arms of hpRNAs have to find their self-complementarity by random, but tethered, collisions. If there is a threshold of duplex RNA required for PTGS in plants, then facilitating more efficient duplex RNA formation from ihpRNA might raise the level in low transgene-expressing plants such that PTGS is enabled. Similar levels of transcription of non-spliced hpRNA might produce lower steady-state levels of duplex RNA that are insufficient for PTGS. The same threshold theory could also be applied for the tighter loop of ihpRNA, giving more nuclease-stable and higher steady-state duplex RNA levels than the larger looped hpRNA. To test the validity of these possibilities, a construct was made in which a spacer region was inserted between one of the arms and the intron in a PVY ihpRNA construct (Figure 1b). This spacer region should impede alignment of the arms during the splicing process and produce a spliced hpRNA with a large loop. When plants transformed with the construct were challenged with PVY, 32 out of 36 independent transformants were immune to the virus. This suggests that the majority of the intron-enhanced silencing efficiency is not due to better alignment of the RNA arms or by presence of a tighter ssRNA loop. It may also explain why the GUS-hpRNA construct so efficiently silenced GUS in

the rice plants reported in Figure 1(a), as this construct contained an intron in the 5' untranslated leader sequence of the ubiquitin promoter.

A common feature of our hpRNA constructs has been the use of matched-length arms. These constructs should produce hpRNA with only a few unpaired 5' nucleotides. If pHANNIBAL is to be used as a generic vector for inserts from gene libraries, occasionally a restriction site within the PCR fragment will be common to the one used to clone into the polylinker. This will sometimes lead to the unintended construction of an hpRNA with unmatched arm length. To investigate whether this was an important attribute, a pHANNIBAL construct was made (using the

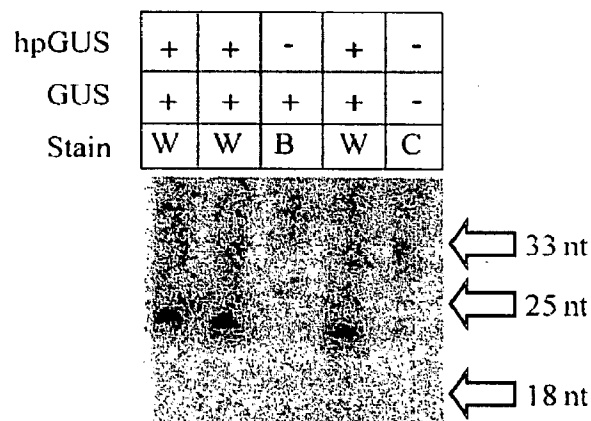


Figure 3. Detection of short (~22 nt) GUS-derived RNAs in tobacco plants showing hpRNA-mediated GUS silencing.

Total RNA (20 µg each), isolated from tobacco plants showing independent segregation of a 35S GUS transgene and a 35S hpGUS transgene, was separated in a 15% denaturing polyacrylamide gel, blotted onto a Hybond-N membrane and hybridized with *in vitro*-transcribed <sup>32</sup>P-labelled GUS RNA. The presence (+) or absence (-) of the target GUS and/or the 35S hpGUS transgene in the plant, from which the sample was taken, is indicated above each track. The phenotype of each of these plants after incubation with X-glucuronide and removal of chlorophyll with ethanol is also indicated. Silenced lines are white (W), unsilenced lines blue (B). The non-transgenic control is designated (C). Sizes indicated on the filter were determined by migration of DNA oligonucleotides.

Figure 2. Silenced phenotypes in ihpRNA transformed plants and recombinase cloning into pHELLSGATE.

- Arabidopsis* seed samples from left to right: five independent CHS-ihpRNA lines; wild-type seed; *tt4*CHS mutant; three CHS-anti-sense lines and three CHS-co-suppression lines. The anti-sense and co-suppression lines were chosen as those showing the lightest seed-coat pigmentation.
- Arabidopsis* seed samples from four independent CHS-ihpRNA lines, chosen to reflect the range of seed-coat pigmentation, viewed under a light microscope.
- Four companion seed samples to (b) from left to right: *tt4*CHS mutant; the two anti-sense-silenced lines from Table 1; and wild-type seed.
- Three pots of *Arabidopsis* plant lines 25 days after germination. From left to right: wild-type; earliest-flowering anti-sense; and FLC1-pHANNIBAL-transformed line.
- Arabidopsis* transformed with EIN2-pHANNIBAL growing on 50 µM ACC. The larger, vigorous plantlet is an ethylene-insensitive EIN2-pHANNIBAL plant; the small plantlets are ethylene-sensitive wild-type plants.
- X-glucuronide-stained transgenic GUS tobacco plantlets segregating for presence (left) or absence (right) of the GUS-hpRNA transgene.
- Agarose gel of restriction enzyme-digested plasmid preparations from nine individual colonies recovered from *E. coli* transformed with a pHELLSGATE/400ntPCR-product recombination reaction. XhoI digestion (g) will release the 400 nt sense arm but not the ccdB fragment, and XbaI digestion (h) will release the 400 nt anti-sense arm but not the other ccdB fragment. Left-hand track in both gels contains size markers.

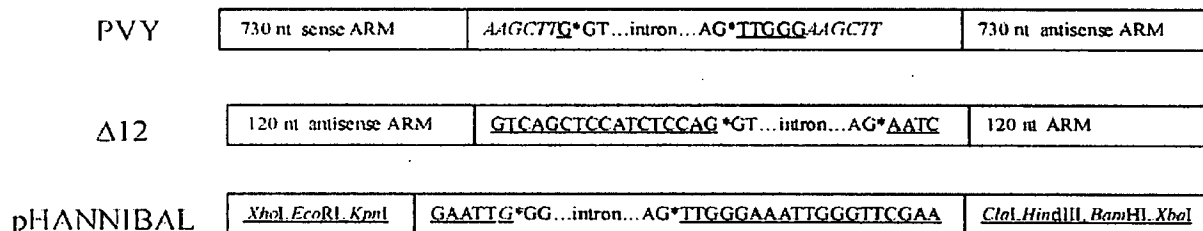


Figure 4. Splice junctions, loop regions and self-complementarity of ihpRNA constructs: PVY,  $\Delta 12$ -desaturase, and pHANNIBAL.

Nucleotides underlined have no complement in the hpRNA and should form a loop structure. \*, splice point; *HindIII* site in PVY-ihpRNA construct shown in italics.

PVY-Pro sequence) that should produce a hpRNA with a stem of 400 nt and 5' region of 300 unpaired nucleotides (Figure 1b). When 48 independent transformed plants, containing this construct, were challenged with PVY, 38 (=80%) of them were immune to the virus. This shows that an unpaired 5' extension of hpRNA does not abolish its ability to induce silencing, although its efficiency may be slightly reduced.

#### High-throughput vector

With the completion of the *Arabidopsis* genome project; the advent of micro-array technology; and the ever-increasing investigation into plant metabolic, perception and response pathways, a rapid, targeted way of silencing genes would be of major assistance. The high incidence and degree of silencing in plants transformed with pHANNIBAL constructs suggest that it could form the basis of a high-throughput silencing vector. However, one of the major obstacles in using pHANNIBAL for a large number of defined genes or a library of undefined genes would be cloning the hairpin arm sequences for each gene in the correct orientations.

Attempts to clone PCR products of sense and anti-sense arms, together with the appropriately cut pHANNIBAL vector as a single-step four-fragment ligation, failed to give efficient or reproducible results (data not shown). Therefore a construct (pHELLSGATE) was made (Figure 5) to take advantage of Gateway technology which facilitates easy cloning of PCR fragments (<http://www.invitrogen.com/content.cfm>). With this technology, a PCR fragment is generated (bordered with recognition sites attB1 and attB2) which is directionally recombined *in vitro* into a plasmid containing attP1 and attP2 sites using the commercially available recombinase preparation.

The pHELLSGATE vector was designed such that a single PCR product from primers with the appropriate attB1 and attB2 sites would be recombined into it simultaneously to form the two arms of the hairpin (Figure 5). The *ccdB* gene, which is lethal in standard *E. coli* strains such as DH5 $\alpha$  (but

not in DB3.1), was placed in the locations to be replaced by the arm sequences, ensuring that only recombinants containing both arms would be recovered. Placing a chloramphenicol-resistance gene within the intron gives a selection to ensure the retention of the intron in the recombinant plasmid. The pHELLSGATE vector was tested using 200 and 400 nt PCR products for two different genes. Many bacterial colonies were obtained on chloramphenicol-containing plates spread with DH5 $\alpha$  bacteria, transformed with the *in vitro* recombination reaction. Analysis of 24 colonies transformed with the 400 nt reaction and 36 colonies from the 200 nt reaction showed that, in both cases, all but one of the colonies contained the desired recombinant plasmid (Figure 2g,h). This was confirmed by sequence analysis (data not shown). These results show that this vector facilitates the rapid, efficient and simple production of hpRNA constructs. pHELLSGATE is a binary vector, with a high-copy-number origin of replication for ease of handling. Recombinant pHELLSGATE constructs can be directly transformed into *Agrobacterium* for transformation into plants. This system should lend itself to high-throughput applications.

#### Discussion

Now that the genomes of a number of species have been completely sequenced, the challenge is to understand the functions and interplay of genes in an organism. The use of chemical mutagens, transposons and T-DNA tagging have been very useful in screening for mutants of individual genes. However, with these undirected methods it is often slow and laborious work to identify each mutant and to track down the gene responsible. RNAi has revolutionized the study of genes in *C. elegans* and *Drosophila*, with two groups recently reporting the systematic analysis of over 4000 genes on chromosomes I and III in *C. elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). By way of comparison, chromosome 2 of *Arabidopsis* has been entirely sequenced (Lin *et al.*, 2000) and the presence of 4037 genes has been predicted. Yet to undertake a systematic analysis

Figure 5. Maps and cloning strategies for pHANNIBAL and pHELLSGATE. PCR products from the target gene are cloned into the polylinkers of pHANNIBAL conventionally; restriction sites added by the primers ensure the correct orientation of the resulting sense and anti-sense arms. The attB1 and attB2 sequences on a single PCR product facilitate the recombination of one sense-orientated and one anti-sense-orientated molecule into each molecule of pHELLSGATE when incubated with BP clonase. The complete sequences and annotations for pHANNIBAL and pHELLSGATE have been lodged at EMBL (Acc No: AJ311872 and AJ311874).

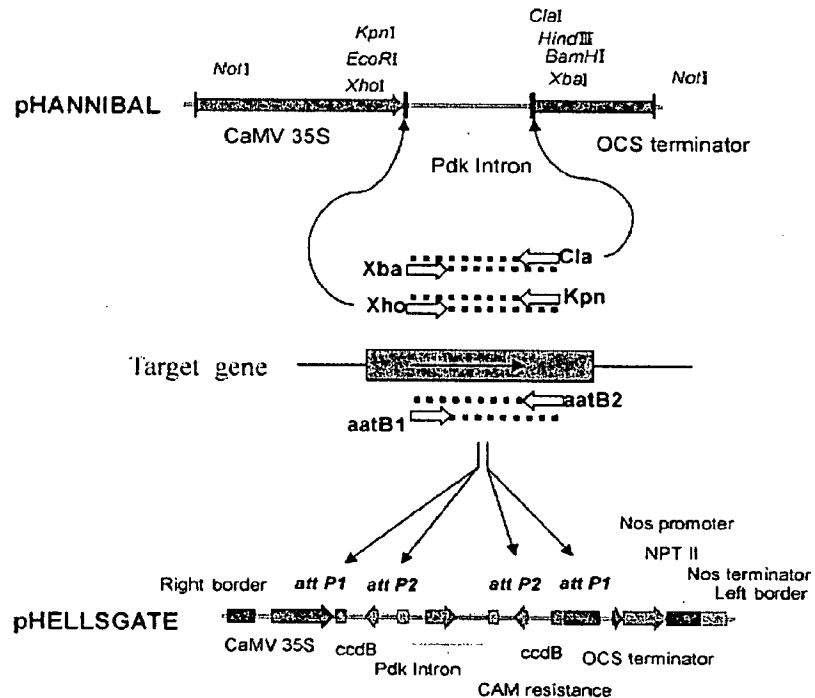


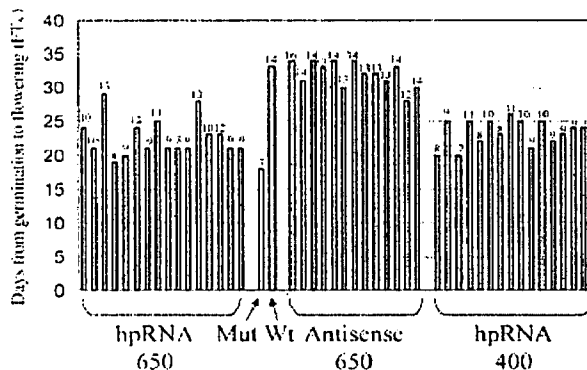
Table 1. Efficiency of hpRNA, co-suppression and anti-sense constructs at silencing a range of genes in a range of plant species

Gene	Species	Prom	Intron	Target	Arm (nt)	GenBank Acc No.	Co-ordinates	ihp RNA	hp RNA	Sense	Anti-sense
PPO	Tobacco	35S	Pdk	ORF	572	AX028815	172-844	21/30		5/54	
GUS	Tobacco	35S		ORF	800	S69414	1-800		23/48		
PVY	Tobacco	35S	Pdk	ORF	730	U09509	6278-7008	23/24	25/43	2/27	1/25
EIN2*	<i>Arabidopsis</i>	35S	Pdk	ORF	600	AF141202	538-1123	42/64			
FLC1*	<i>Arabidopsis</i>	35S	Pdk	ORF	650	AY034083	1-650	16/16			2/13
FLC1*	<i>Arabidopsis</i>	35S	Pdk	ORF	400	AY034083	250-650	15/15			
CHS	<i>Arabidopsis</i>	35S	Pdk	ORF	741	AF112086	248-1075	21/23		2/19	0/25
Δ12	<i>Arabidopsis</i>	Napin	Δ12a	3' UTR	120	L26296	1243-1363	30/30	44/63	4/41	3/21
Δ12	Cotton	Lectin		ORF	853	X97016	68-921		17/29		7/30
Δ12	Cotton	Δ12c	Δ12c	5' UTR	98	X97016	1-98	26/26			
Δ9	Cotton	Lectin		ORF	514	X95988	24-538		15/26		4/30
GUS	Rice	Ubi		ORF	560	S69414	1-560	12/14		3/10	1/8
Average percentage of silenced plants								90	58	13	12

The type of promoter (Prom), type of intron, length of arms and details of how to find the specific sequences of the arms for various gene-silencing constructs are shown. The last four columns show the number of primary independent transformants (or transformed lines where progeny were analysed) showing silencing/the number of transgenic plants produced from the primary transformation experiment. \*Silencing analysis was done on the progeny of the primary transformed plants.

of these genes using the conventional plant technologies of insertional mutagenesis would require vast resources. It has been calculated that to have a 90% chance of finding just one specific single gene (of about 1 kb) in *Arabidopsis* using T-DNA insertional mutagenesis would require the generation of about 350 000 independent transformants

(Krysan *et al.*, 1999). The work described in this paper facilitates a directed silencing which, when combined with the efficient, non-tissue culture transformation method for *Arabidopsis* (Clough and Bent, 1998), provides the tools that make the challenge of mirroring in plants, the gene discovery under way in nematodes, more feasible.



**Figure 6.** Flowering time in transgenic progeny from independent FLC1-pHANNIBAL and FLC1-anti-sense transformed C24 *Arabidopsis* lines. Flowering time (FT<sub>50</sub>) and leaf number (in figures above each column) for transgenic progeny from 16 and 15 independent plant lines transformed with pHANNIBAL constructs containing 650 and 400 nt arms of FLC1 sequence, respectively; 13 independent plants transformed with a conventional, 35S-driven, anti-sense construct containing the appropriate 650 nt of FLC1; the flc-13 mutant (white bar); and wild-type plants (cross-hatched bar). Wild-type plants were transgenic for GUS and kanamycin resistance, to allow the plants to be grown under identical conditions. The standard error for leaf numbers did not exceed 0.7 of 1 day for any plant line.

Using hpRNA constructs, we have obtained silenced plants for every gene that we targeted, irrespective of whether it was a viral gene, transgene or endogenous gene, and the silencing appears to be uniform within tissues in which the hpRNA is expressed. With ihpRNA constructs the efficiency averaged about 90%, and arms of 400–800 nt appear to be stable and effective. High levels of silencing were obtained with constructs having unmatched arm lengths, with arms as long as 853 nt or as little as 98 nt, and with arm sequences derived from coding, 3' or 5' untranslated regions of the target gene. These results suggest that ihpRNA constructs will be effective in a wide range of circumstances, and augur well for the generic use of the technology. The silencing was much more profound with ihpRNA constructs than either anti-sense or co-suppression constructs; some ihpRNA transformants were close to exhibiting a complete knock-out of the target endogenous gene. However, most of the ihpRNA plants showed dramatically reduced but detectable levels of target gene activity. This variation in degree of silencing in the ihpRNA plants may be a useful feature for gene discovery and genomics: complete silencing of genes required for basic cell function or development will probably be embryo-lethal and therefore not easily recovered using traditional tagging approaches, whereas the reduced gene expression caused by hpRNA constructs may give viable plants with phenotypes indicative of the role of the target gene.

Although the pHANNIBAL construct should be very useful for studying a modest number of genes (e.g. 10–

50), such as in a metabolic pathway, it would not be feasible with normal resources to use it for hundreds to thousands of genes. However, the pHELLSGATE vector has the potential to facilitate making large numbers of gene ihpRNA constructs rapidly and efficiently. The simple steps required, namely PCR, incubation of the PCR product with the vector and recombinase, selection of recombinant plasmid, and then transformation into *Agrobacterium*, are steps that could easily be automated. The templates for PCR could be the defined genes in an EST library using standard forward and reverse primers. Alternatively, given that ihpRNA constructs with arms as small as 98 nt give effective silencing, oligosynthesizers could be automated to systematically synthesize oligonucleotides of each computer-identified gene along a chromosome, or for genes for which no function is known, and pass these primers into an automated ihpRNA production system.

It has been shown that RNAi in *Drosophila* is directed by 21 nt dsRNA oligomers derived from the inducing dsRNA (Elbashir *et al.*, 2001; Zamore *et al.*, 2000). Similar 21–25 nt RNAs have also been found associated with PTGS in plants (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001). It is tempting to speculate that the minimum region of homology between an mRNA and the arms of an effective hpRNA will also be 21–25 nt. If so, this rule would allow the design of hpRNAs to silence a single member of a gene family, as such unique sequences are present in most gene families. Also, by choosing conserved regions, it may be possible to silence whole gene families using a single construct. However, these rules remain to be proven.

## Experimental procedures

### Plasmid construction

Standard gene cloning methods (Sambrook *et al.*, 1989) were used to make the gene constructs. The plasmids for dicot transformation were derived from pART7 and pART27 (Gleave, 1992), and those for monocot transformation were derived from pVec4 (Wang *et al.*, 1997; Wang *et al.*, 1998). The accession numbers of the gene sequences, and the co-ordinates of the sequences used in the hpRNA, co-suppression and anti-sense constructs, are shown in Table 1. The annotated sequences of pHANNIBAL, pKANNIBAL and pHELLSGATE are lodged with EMBL and have accession numbers AJ311872, AJ311873 and AJ311874, respectively. Constructs made in pHANNIBAL were subcloned as *NotI* fragments into pART27, then introduced into *Agrobacterium* strains AGL1 or LBA4404 either by electroporation or tri-parental mating. pHELLSGATE was maintained in *E. coli* strain DB3.1 (Invitrogen, Carlsbad, CA, USA) in which the *ccdB* gene is not lethal.

### Plant transformation

*Nicotiana tabacum* (W38), cotton and rice were transformed essentially as described by Ellis *et al.* (1987), Cousins *et al.* (1991) and Wang *et al.* (2001), respectively. *Arabidopsis* was transformed by the dipping method of Clough and Bent (1998).

### Analysis of transgenic plants

Northern blot analysis for the presence or absence of short RNAs was performed essentially as described by Wang *et al.* (2001).

Polyphenol oxidase (PPO) activity was measured using an oxygen electrode essentially as described by Robinson and Dry (1992). Rice and tobacco were tested for GUS activity using the histochemical stain X-glucuronide essentially as described by Jefferson *et al.* (1987). The reactions of plants to potato virus Y were analysed as described by Waterhouse *et al.* (1998). The activity of EIN2, which is required in the ethylene perception pathway, was observed by growing plants on media containing 1-aminocyclopropane-1-carboxylic acid (ACC) as described by Alonso *et al.* (1999). To identify lines silenced for EIN1, at least 30 progeny of each transformed line were germinated and grown on ACC-containing media.

To measure the effect of silencing FLC1, 20–30 seeds from each transgenic C24 *Arabidopsis* line, the transposon mutant flc13 (Sheldon *et al.*, 2000), and a control GUS line, were germinated and grown on kanamycin plates as described by Sheldon *et al.* (2000). The plants were scored daily over a 40-day period for the appearance of flowers. Flowering time (FT<sub>50</sub>) for each line was taken as the number of days after germination for 50% of the plants to show flowering. After flowering, the number of leaves of 10 randomly selected plants was counted for each line.

Chalcone synthase (CHS) activity was monitored by visual observation of stem and leaf colour in plants grown under high light, and by unaided or microscope-assisted visual observation of seed-coat colour. The seeds were collected after they had matured and dried on the plant. The relative flavonoid concentrations in seeds were determined by measuring extracts for absorbance between 490 and 530 nm in a Spectramax 340-PC (Molecular Devices Corporation, Sunnyvale, CA, USA). Duplicate extracts were made from 25 mg seed of each line, essentially as described by Gerats *et al.* (1982). The average absorbance value for each line was mathematically transformed to give relative values such that the tt4 and wild-type seed became values of 0 and 100%, respectively.

The activity of  $\Delta 12$ - and  $\Delta 9$ -desaturase activity during lipid synthesis was estimated from the relative proportions of individual fatty acids in mature seed, as determined by routine methods for GC analysis of fatty acid methyl esters.

Unless otherwise stated, plants were considered to be showing silencing when they showed obvious appropriate phenotypic differences from wild-type plants, or when they had a gene activity that was reduced by at least 20%.

### AttB primers, PCR and recombination reaction for introduction of sequences into pHELLSGATE

Primers with attB1 and attB2 sequences were purchased from Life Technologies. Polymerase chain reactions (PCR) and *in vitro* BP clonase recombination reactions were carried out according to the manufacturer's instructions (Invitrogen). The recombination reaction product was either electroporated or heat-shocked into RbCl-treated DH5 $\alpha$  *E. coli*.

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## Double Jeopardy: Both Overexpression and Suppression of a Redox-Activated Plant Mitogen-Activated Protein Kinase Render Tobacco Plants Ozone Sensitive

Marcus A. Samuel and Brian E. Ellis<sup>1</sup>

Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, V6T 1Z3 Canada

In plants, the role of mitogen-activated protein kinase (MAPK) in reactive oxygen species (ROS)-based signal transduction processes is elusive. Despite the fact that ROS can induce MAPK activation, no direct genetic evidence has linked ROS-induced MAPK activation with the hypersensitive response, a form of programmed cell death. In tobacco, the major ROS-induced MAPK is salicylate-induced protein kinase (SIPK). We found through gain-of-function and loss-of-function approaches that both overexpression and RNA interference-based suppression of SIPK render the plant sensitive to ROS stress. Transgenic lines overexpressing a nonphosphorylatable version of SIPK were not ROS sensitive. Analysis of the MAPK activation profiles in ROS-stressed transgenic and wild-type plants revealed a striking interplay between SIPK and another MAPK (wound-induced protein kinase [WIPK]) in the different kinotypes. During continuous ozone exposure, abnormally prolonged activation of SIPK was seen in the SIPK-overexpression genotype, without WIPK activation, whereas strong and stable activation of WIPK was observed in the SIPK-suppressed lines. Thus, one role of activated SIPK in tobacco cells upon ROS stimulation appears to be control of the inactivation of WIPK.

### INTRODUCTION

Mitogen-activated protein kinase (MAPK) modules form a key part of the eukaryotic signal transduction network that links environmental inputs to a wide range of modifications of cellular functions, ranging from cell division to cell death. In plants, MAPK signaling has been implicated in defense against pathogens and herbivores, in cellular responses to auxin, abscisic acid, and other phytohormones, in cell cycle control, in the induction of programmed cell death, and in responses to abiotic stresses such as UV light and ozone (Zhang and Klessig, 1997; Kovtun et al., 1998; Romeis et al., 1999; Heimoavaara-Dijkstra et al., 2000; Samuel et al., 2000; Nishihama et al., 2001; Yang et al., 2001; Miles et al., 2002).

A variety of stress responses have been found to involve the rapid activation of a specific subset of plant MAPKs, notably *Arabidopsis* MPK6 (Ichimura et al., 2000; Kovtun et al., 2000; Nühse et al., 2000; Yuasa et al., 2001) and its orthologs in other species, such as salicylic acid-induced protein kinase (SIPK) in tobacco (Zhang and Klessig, 1998a, 1998b; Romeis et al., 1999; Mikolajczyk et al., 2000; Samuel et al., 2000; Zhang et al., 2000) and salt stress-induced MAPK (SIMK) in alfalfa (Cardinale et al., 2000). Because many biotic

and abiotic stressors (virus infection, treatment with microbial elicitors, wounding, and osmotic stress) elicit a very rapid oxidative burst in plant cells, the apparent convergence of disparate stress signals on this particular MAPK node may be related to the sensitive response of MPK6/SIPK to redox perturbation.

Exposure to ozone immediately creates an oxidizing environment in plant tissues and triggers an array of cellular responses, including the accumulation of antioxidants, elicitation of pathogenesis-related proteins, deposition of phenols, induction of ethylene synthesis, suppression of primary metabolic activities such as photosynthesis, and eventually cell death (Darrall, 1989; Schraudner et al., 1992; Conklin and Last, 1995; Sharma and Davis, 1997; Tuomainen et al., 1997). Ozone enters the plant mesophyll through the stomata and diffuses through inner air spaces. In the cell wall and plasmalemma, it is converted spontaneously to reactive oxygen species (ROS) by contact with either water or membrane components (Sharma and Davis, 1997). The ozone-induced cell death process is influenced by the interaction of multiple signaling molecules, including salicylic acid, jasmonic acid, and ethylene (Orvar and Ellis, 1997; Overmyer et al., 2000; Rao et al., 2000).

One of the earliest responses elicited by ozone and other ROS generators in plants is the activation of specific MAPKs (Samuel et al., 2000; Desikan et al., 2001). The primary ROS-activated tobacco MAPK has been identified as the 46-kD

<sup>1</sup>To whom correspondence should be addressed. E-mail bee@interchange.ubc.ca; fax 604-822-2114.  
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SIPK; a second MAPK, the 44-kD wound-induced protein kinase (WIPK), usually responds more weakly (Kumar and Klessig, 2000; Samuel et al., 2000).

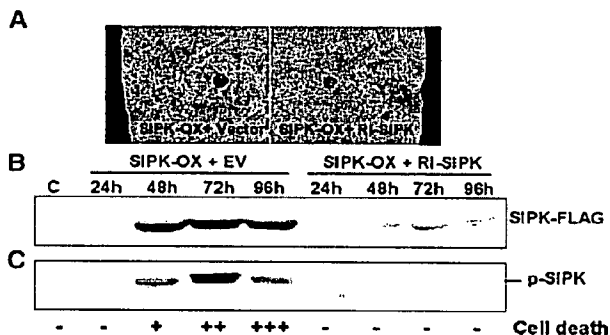
The rapid activation of these MAPKs suggests that their action on downstream targets could be important for the modulation of the cellular response to increased oxidative damage, but direct evidence for that role is lacking in plants. No intracellular substrates have been identified for either SIPK or WIPK, nor have loss-of-function genotypes been assessed for their ability to control redox stress. Stable overexpression or suppression of SIPK or WIPK in transgenic tobacco apparently did not result in the alteration of its activity (Yang et al., 2001). By contrast, transient overexpression of SIPK or its upstream activator, NtMEK2, in an active form has been shown to lead to the activation of either SIPK or both SIPK and WIPK, with associated induction of defense genes and hypersensitive response (HR)-like cell death (Yang et al., 2001; Zhang and Liu, 2001). This finding suggests that SIPK may play a role as a positive regulator in the cell death pathway.

The previously reported inability to produce SIPK-suppressed lines, and the lack of phenotype or alteration of SIPK activity reported for overexpression lines (Yang et al., 2001), have suggested that the normal functioning of this kinase may be essential for cell survival. However, we report here, using RNA interference (RNAi) technology, the recovery and analysis of transgenic tobacco plants in which SIPK is either overexpressed ectopically or largely eliminated. These plants display distinctive ozone response phenotypes that confirm the importance of SIPK activation for the effective control of ROS damage and also reveal an unexpected interplay between the activities of SIPK and WIPK.

## RESULTS

Infiltration of fully grown tobacco leaves with a suspension of *Agrobacterium tumefaciens* cells carrying a SIPK-FLAG overexpression construct resulted in the accumulation of the epitope-tagged SIPK protein in the infiltrated tissue within 48 h. In unstressed cells, endogenous SIPK was not phosphorylated at the TXY motif found in the activation loop of the kinase, as indicated by the absence of any signal in the control lane of a protein gel blot (Figure 1C) prepared using an anti-pMAPK antibody that specifically recognized the doubly phosphorylated protein. In the infiltrated tissue, however, at least a portion of the pool of SIPK became activated by 48 h after infiltration, with even greater activation observed by 72 h. In the same period, the infiltrated zones showed signs of tissue collapse, and by 96 h, these zones became completely necrotic (Figure 1A).

When leaves were coinfiltrated with *Agrobacterium* carrying the SIPK-FLAG overexpression construct plus an RNAi construct that targeted SIPK, both expression and activation of SIPK-FLAG were suppressed completely (Figures 1B and



**Figure 1.** Post-Transcriptional Gene Silencing-Induced Suppression of Cell Death Triggered by Transient Overexpression of SIPK.

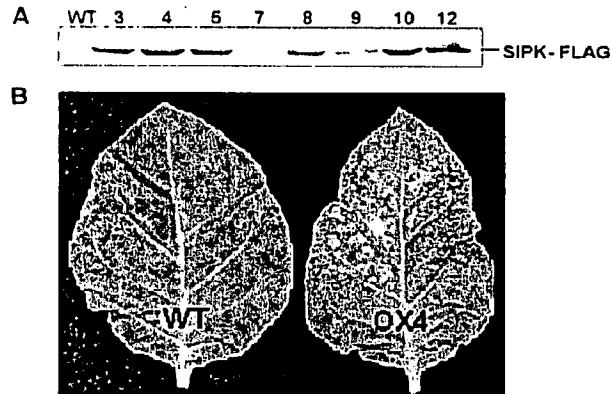
Coinfiltration with *Agrobacterium* containing the SIPK-FLAG construct along with the SIPK-RI construct inhibited the cell death process induced by transient overexpression of SIPK-FLAG alone (A). Protein samples extracted at different times after infiltration of the constructs were immunoblotted with either anti-FLAG antibody (B) or phospho-MAPK-specific antibody (anti-pERK) (C). -, +, ++, and +++ indicate the extent of visible lesions appearing in the infiltrated zones. EV, *agrobacterium* carrying empty vector.

1C). The cell death induced by the overexpression of SIPK-FLAG in the infiltrated zones also was eliminated (Figure 1A).

The cell death associated with the spontaneous activation of SIPK in overexpression (OX) transgenic cells suggested that it might be difficult to recover stably transformed lines using this construct, but cocultivation of tobacco leaf discs with the appropriate *Agrobacterium* culture and selection on kanamycin yielded a number of transgenic lines that were found to ectopically express a range of levels of SIPK-FLAG (Figure 2A). No spontaneous activation of SIPK was detected in these lines, all of which displayed normal growth and development phenotypes.

Transformation of tobacco leaf discs with the SIPK-RI construct also yielded stable transgenic lines, although with a sharply reduced frequency. In the recovered RI lines, silencing of endogenous SIPK expression was observed to varying degrees, ranging from partial reduction in both SIPK mRNA and protein to elimination of both products (Figures 3B and 3C). The specificity of this silencing was shown by the continued expression in most of the recovered RI lines of the closely related *NTF4* MAPK gene, whose cDNA sequence is 89% identical to that of *SIPK* (Figure 3D). The RI lines again showed largely normal growth and development phenotypes, although the most severely suppressed lines showed some modest tendency to dwarfing (data not shown).

Plants of both the OX and RI lines showed no signs of spontaneous cell death under normal growth conditions. However, exposure of mature OX or RI leaves to levels of ozone that caused no visible injury to wild-type plants (500 parts per billion [ppb]) resulted in the rapid appearance of small necrotic lesions on leaves of both the transgenic



**Figure 2.** Transgenic Tobacco Plants Overexpressing SIPK-FLAG Show Increased Ozone Sensitivity.

(A) Proteins (40  $\mu$ g) extracted from leaves of the different OX lines ectopically expressing SIPK-FLAG were immunoblotted using anti-FLAG antibody.

(B) Transgenic tobacco line OX4 and wild-type tobacco (Xanthi-nc) plants ( $n = 25$ ) were exposed to ozone (500 ppb) for 8 h. The treated leaves were photographed 24 h after exposure. WT, wild type.

genotypes (Figures 2B and 3E). The kinetics of this oxidative stress damage were quite different. Lesions consistently appeared on the leaves of OX plants as early as 4 to 6 h, but visually similar lesions only appeared on RI leaves ~24 h later. In plants challenged with lower ozone concentrations (250 ppb), an analogous pattern was observed except that the necrotic responses were delayed until 48 h (OX) and 72 h (RI) (data not shown). When leaf discs prepared from the wild-type, OX, and RI genotypes were assayed for the loss of membrane integrity and associated ion leakage resulting from ozone exposure (500 ppb), differential timing of the damage response also was observed (Figure 4).

To assess *in situ* the relative levels of hydrogen peroxide accumulation induced by ozone exposure, control and ozone-treated leaf halves were infiltrated with 3,3'-diaminobenzidine solution. The staining patterns revealed no detectable levels of hydrogen peroxide in untreated leaves of any of the genotypes or in leaves of wild-type plants after 8 h of ozone exposure. However, strong 3,3'-diaminobenzidine staining was observed in both the OX and RI lines after ozone treatment (Figure 4B).

The observation that overexpression of SIPK-FLAG in infiltrated leaves was accompanied by the spontaneous activation of MAPK and by cell death raised the question of whether activation of the ectopically expressed protein was necessary for the induction of cell death. Therefore, site-directed mutagenesis was used to create a version of SIPK-FLAG in which the TEY motif found in the activation loop of SIPK had been converted to an AEF sequence. This modifi-

cation yielded a kinase that retained a low level of basal activity when the recombinant protein was assayed *in vitro* against myelin basic protein (Figure 5A), but it could not be activated further through dual phosphorylation of the activation loop by upstream MAPK kinases. Unlike the SIPK-FLAG construct, when transiently expressed in tobacco leaves, the SIPK(AEF)-FLAG construct failed to cause cell death in the infiltrated zone (data not shown).

Stably transformed tobacco plants expressing high levels of SIPK(AEF)-FLAG also were recovered readily after *Agrobacterium* cocultivation, and these plants displayed no visibly altered phenotype. Despite accumulating similar levels of the epitope-tagged kinase (Figure 5B), the ozone sensitivity of these SIPK(AEF) transgenic lines did not differ from that of wild-type plants (data not shown). This finding indicates that the heightened ozone sensitivity observed in SIPK-OX transgenic lines requires not only that the ectopically expressed kinase be expressed at high levels within the plant cell but that it have the capacity to become activated.

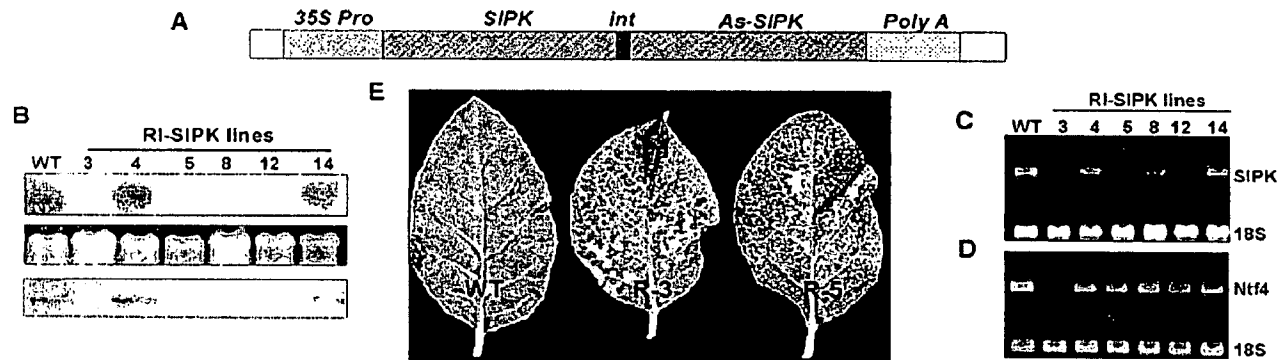
The activation status of both SIPK and WIPK in tobacco tissue extracts can be assessed either on protein gel blots using a phosphospecific antibody or by immunoprecipitation with antibodies that discriminate between SIPK and WIPK, followed by *in gel* or *in vitro* kinase activity assays. When the various transgenic and wild-type tobacco lines were monitored during a 30-min period of ozone exposure, striking differences in the pattern of kinase activation were observed among these genotypes (Figure 6).

As reported previously (Samuel et al., 2000), ozone treatment led to the rapid activation of SIPK in leaves of wild-type plants. This was accompanied by a much weaker activation of the smaller kinase, WIPK (Figure 6A). In the OX4 genotype, ozone exposure also led to SIPK activation, but the level of activation appeared to be depressed relative to the wild-type response, despite the presence of far greater amounts of ectopically expressed SIPK in the OX cells (Figures 6B and 6C). No activation of WIPK was detected in the OX tissue samples.

The SIPK(AEF) genotype presented a kinase activation profile that was very similar to that of the wild type. This indicates that flooding the cell with a nonactivatable version of SIPK (a potential dominant-negative form) does not interfere with the ability of the upstream MAPK cascade elements to transmit oxidant-induced signals to their cognate MAPKs.

Exposure of the RI genotype to ozone, on the other hand, yielded a very different MAPK activation profile. Very weak or no SIPK activation was detected, as would be predicted for a genotype in which SIPK expression has been suppressed by post-transcriptional gene silencing (Figures 6A and 6C). Instead, ozone exposure produced strong and specific activation of WIPK. The identity of these highly activated kinases in ozone-treated leaves of each genotype was confirmed through immunoprecipitation of the 30-min ozone-treated protein extracts with either SIPK- or WIPK-specific antibodies, followed by *in gel* kinase assays (Figures 6D and 6E).

Aside from the unexpected massive activation of WIPK, the stability of that activation also was strikingly different in



**Figure 3.** SIPK-Suppressed Lines Also Are Sensitive to Ozone.

(A) RNAi construct under the control of the 35S promoter of *Cauliflower mosaic virus*.

(B) SIPK is suppressed in four of the six PCR-positive lines. RNA gel blot analysis was performed using total RNA (15  $\mu$ g) extracted from wild-type and SIPK-RI lines and probed with the radiolabeled C-terminal fragment of the SIPK ORF. Autoradiography revealed essentially no SIPK mRNA in four of the six PCR-positive lines (top). Ethidium bromide staining of the gel showed equal loading of RNA (middle). Immunoblot analysis of protein samples from the same lines indicated the absence of detectable amounts of SIPK protein in all four SIPK-suppressed lines (bottom).

(C) Similar results were observed when reverse transcriptase-mediated PCR was conducted using SIPK-specific primers.

(D) *Ntf4* gene expression in the SIPK-suppressed lines was analyzed by reverse transcriptase-mediated PCR using gene-specific primers.

(E) SIPK-suppressed lines R3 and R5 display ozone-sensitive phenotypes. Plants ( $n = 15$ ) of SIPK-suppressed tobacco transgenic lines R3 and R5, together with wild-type plants, were exposed to ozone (500 ppb) for 8 h per day for 2 days. The treated leaves were photographed 24 h after the end of 2 days of exposure.

WT, wild type.

this genetic background. Normally, when oxidants trigger a rapid activation of SIPK, it is a transient response. The activation is effectively lost within 1 h, even under conditions of continuous oxidant stimulus, as seen in Figure 7A (wild-type lane). However, in the RI genotype, WIPK was not only activated rapidly but the pool of this MAPK remained continuously active for up to 8 h after the initiation of the response (Figure 7A, RI lane). Although normally there is far less WIPK than SIPK present in tobacco leaves (Zhang and Klessig, 1998b), the high activation signal observed in the RI tissue extracts did not appear to reflect increased levels of WIPK protein in this genotype compared with wild-type plants, as assessed by protein gel blot analysis (Figure 7C).

Interestingly, kinase activation by ozone in the OX genotype also was prolonged abnormally, relative to that seen in ozone-treated wild-type plants, but in this case, the active kinase was SIPK rather than WIPK (Figure 7B). In addition, unlike the hyperactivated WIPK pool, the extended activation of SIPK in the OX line was more transient and disappeared within 4 h. This is approximately the time at which visible lesions began appearing on ozone-treated OX leaves.

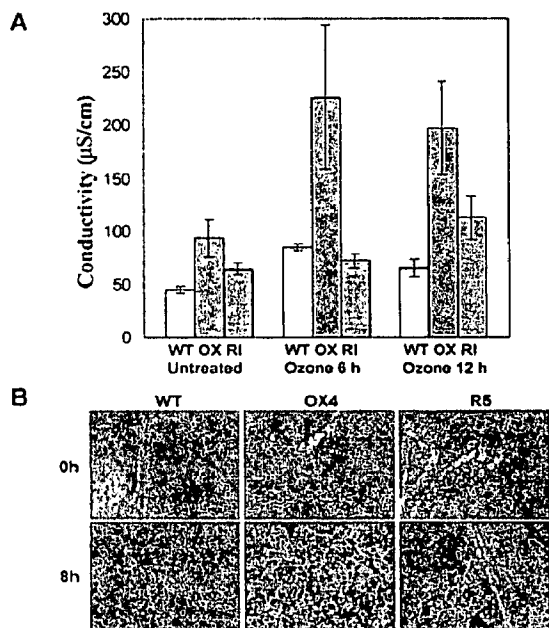
Examination of the temporal response of the two genes (*GST* [glutathione S-transferase] and *cAPX* [cytosolic ascorbate peroxidase]) whose expression was induced strongly by ozone treatment revealed that the loss of SIPK signaling in the RI genotype resulted in a delayed response in the expression of both genes. In the OX line, the prolonged activa-

tion of SIPK signaling resulted in the suppression of *GST* induction, whereas *APX* gene expression was unaffected (Figures 8A and 8B).

## DISCUSSION

Plant cells must deal constantly with ROS from a range of sources, including photooxidation, mitochondrial electron transport, flavin oxidase by-products, and environmental insults such as UV light, ozone, and ionizing radiation. Against this background, ROS pulses ("oxidative bursts") also can occur within cells, usually as very early responses to localized challenges to cellular integrity such as wounding and pathogen assault. These pulses may serve in multiple functions, including activation of redox protection mechanisms, modulation of intracellular signal transduction pathways, and transmission of systemic signals to neighboring cells.

A severe oxidative challenge that overwhelms local protective measures ultimately will lead to cell death. The archetype for this outcome is the HR response induced during incompatible host-pathogen interactions. Similar lesions are induced by exposure to increased levels of ozone or UV light. The exact process by which cellular integrity fails is unclear, but the notion that HR represents a form of genetically programmed cell death is supported by the identification of nu-



**Figure 4.** Quantitation of Ozone-Induced Cell Death and Hydrogen Peroxide Accumulation in SIPK Kinotypes.

(A) Ion leakage from leaf discs (five each) of the third and fourth leaves of wild-type, OX4, and RI5 lines was assessed as an indicator of the loss of membrane integrity 6 and 12 h after the initiation of ozone exposure (500 ppb). The data presented are means and standard deviations from three independent experiments.

(B) 3,3'-Diaminobenzidine staining to detect hydrogen peroxide accumulation in ozone-treated leaves of SIPK kinotypes. WT, wild type.

merous mutants affected in the process of lesion formation (Richberg et al., 1998).

The correlation of ROS pulses with the cell death process has been described extensively. Treatments such as chilling, wounding, pathogen infection, UV irradiation, and ozone exposure rapidly induce ROS accumulation in plant cells, followed later by lesion development. However, despite these correlative observations, a functional link between ROS accumulation and local lesion formation has yet to be defined.

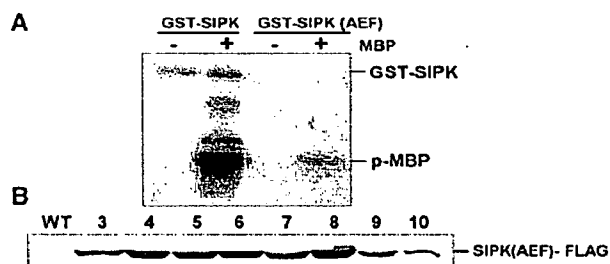
It is striking that so many stresses that elicit ROS accumulation in plant cells consistently appear to activate MAPK modules as one of their earliest effects (Seo et al., 1995; Zhang and Klessig, 1998b; Allan et al., 2001; Desikan et al., 2001; Orozco-Cardenas et al., 2001). The MAPK observed most consistently to be activated by both applied stresses and ROS is SIPK in tobacco (Samuel et al., 2000; Miles et al., 2002) or its apparent orthologs in other species, such as MPK6 in Arabidopsis (Kovtun et al., 2000; Yuasa et al., 2001) and SIMK in alfalfa (Cardinale et al., 2000). This pattern suggests that

SIPK activation might play an important role in determining the response and ultimate fate of the stressed cells.

Links between ROS-associated cell death and MAPK signaling have been reported for a number of nonplant systems. Hydrogen peroxide-induced cell death in cultured mammalian oligodendrocyte cells is inhibited by PD98059, a specific inhibitor of MEK, the upstream kinase of the ERK1/2 MAPK (Bhat and Zhang, 1999), whereas delayed and prolonged activation of p44 and p42 MAPKs is critical for genistein-induced programmed cell death in rat primary cortical neurons (Linford et al., 2001). Similarly, delayed and persistent activation of ERK1/2 is associated with glutamate-induced oxidative cytotoxicity in neuronal cell lines (Stanciu et al., 2000).

There also is evidence that ROS-activated MAPKs may play analogous roles in plant cells. Cell death induced in Arabidopsis cell suspension cultures by treatment with a bacterial elicitor (harpin) is inhibited when the cells are treated with the MEK inhibitor PD98059 (Desikan et al., 1999), whereas pretreatment of tobacco cells with staurosporine, a general protein kinase inhibitor, suppresses the cell death normally induced by exposure to fungal elicitors (Suzuki et al., 1999).

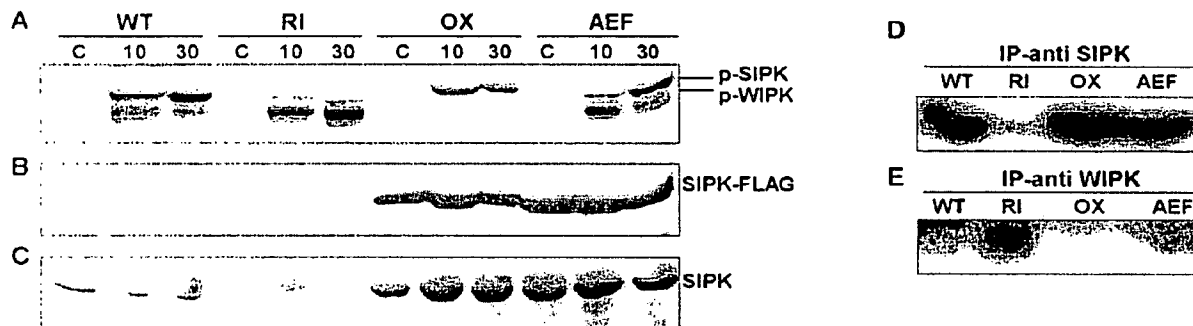
Genetic manipulation experiments also have implicated MAPK activation in the cell death process. In Arabidopsis plants overexpressing constitutively active forms of the MAPK kinases AtMEK4 and AtMEK5 under the control of an inducible promoter, HR-like lesions appeared after induction with dexamethasone, and lesion formation was preceded by the activation of endogenous MAPKs and the accumulation of hydrogen peroxide (Ren et al., 2002). Transient overexpression of a constitutively active form of a MAPK kinase (NtMEK2) in tobacco also led to the sustained activation of MAPKs, identified



**Figure 5.** Activity and Expression of Mutagenized SIPK.

(A) The recombinant SIPK activation loop mutant is less active than wild-type SIPK. Myelin basic protein (MBP)-phosphorylating activities of SIPK and SIPK(AEF) were measured by incubating recombinant proteins (5 µg) with 5 µg of MBP, as described in Methods. The phospho-MBP product was visualized through autoradiography after SDS-PAGE fractionation.

(B) SIPK(AEF) transgenic lines show high expression of the transgene product. Proteins (40 µg) extracted from the different lines overexpressing SIPK(AEF)-FLAG were fractionated by SDS-PAGE and immunoblotted using anti-FLAG antibody. WT, wild type.



**Figure 6.** Differential Ozone-Induced Activation of SIPK and WIPK in SIPK Kinotypes.

(A) Crude protein extracts prepared from ozone-exposed tissues from T1 lines of the different SIPK kinotypes (RI5, OX4, and AEF8) and the wild type were resolved on a 10% polyacrylamide gel, blotted, and probed with an anti-phospho-ERK antibody to recognize phospho-MAPK forms.

(B) The same blot was probed subsequently using an anti-FLAG antibody to detect ectopic expression of the transgene product in the different kinotypes.

(C) A replicate protein gel blot was analyzed using a SIPK-specific antibody, revealing high expression of SIPK forms in the overexpressor lines and its absence in the SIPK-suppressed lines.

(D) and (E) Protein samples prepared from ozone-exposed (30 min) tissues from the different kinotypes were immunoprecipitated with either SIPK-specific (D) or WIPK-specific (E) antibodies. The immunoprecipitates were subjected to an in gel kinase assay, as described in Methods. WT, wild type.

as SIPK and WIPK, and to the death of the infiltrated tissue (Yang et al., 2001). Transient overexpression of SIPK itself was shown subsequently to result in the formation of HR-like lesions, but only in young leaves (Zhang and Liu, 2001).

We have confirmed that ectopic SIPK overexpression leads to the appearance of high levels of the activated kinase in *Agrobacterium*-infiltrated tobacco tissue and to rapid cell death (Figure 1). On the other hand, when stably transformed tobacco plants were produced that overexpressed epitope-tagged SIPK (Figure 2), they displayed no visible phenotype. When exposed to ozone, however, the transgenic SIPK-OX plants proved to be much more sensitive than the nontransgenic parental line, indicating that ROS-induced cell death was controlled less effectively in the overexpression genotype.

Although this pattern is consistent with the results of NtMEK2 or SIPK-OX transient expression, its physiological relevance remains uncertain, because we know little about the effects of the accumulation of nonphysiological levels of active signal components on cellular function. To unambiguously identify a functional relationship between ROS activation of SIPK and ROS-induced cell death, we turned to the creation of defined loss-of-function mutants.

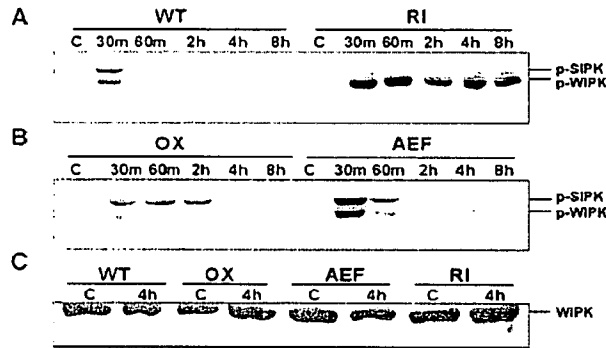
The modification of SIPK function in transgenic tobacco plants using either conventional gene-silencing methods (co-suppression and antisense-mediated suppression) or overexpression of dominant-negative forms proved ineffective (Yang et al., 2001) (data not shown). However, expression of an intron-containing "hairpin RNA" (Smith et al., 2000) designed to target a unique tract within the SIPK coding sequence yielded a number of transgenic plants in which SIPK expression was sup-

pressed severely and specifically through post-transcriptional gene silencing. Loss of SIPK had no obvious phenotypic consequences for plants grown under normal greenhouse conditions.

Given the sensitivity of SIPK-OX lines to ozone, it might have been predicted that the absence of this kinase would have no effects, or perhaps even positive effects, on the ozone sensitivity of the SIPK-RI lines. Instead, after ozone treatments that induced no visible damage on wild-type plants, the SIPK-RI lines developed numerous lesions on their middle leaves within 24 h. Thus, the inability of the suppressed genotype to generate and activate SIPK compromises the cell's ability to manage ROS stress and to control cell death, although apparently on a different time scale from that observed in SIPK-OX plants.

Which facet of ROS-stress management has been compromised in SIPK-OX and SIPK-RI plants is not clear. No constitutive hydrogen peroxide accumulation was detected in any of the genotypes, suggesting that their heightened ozone sensitivity is not the consequence of a preexisting accumulation of ROS. Instead, it appears that alteration of the normal ozone-induced MAPK activation process, through either unregulated overexpression or suppression, creates an inability to cope with increased redox stress. Examination of the transcriptional activity of two genes whose mRNAs accumulate rapidly after ozone exposure showed that the response of both genes was affected differently (Figure 8).

Expression of *cAPX*, which encodes a major ROS-scavenging enzyme, was induced less effectively by ozone in RI plants, whereas it was unaffected in the OX line. Antisense suppression of *cAPX* was shown previously to create hypersensitivity to both ozone (Orvar and Ellis, 1997) and pathogens (Mittler



**Figure 7.** Loss of SIPK Has Differential Effects on the Expression of Ozone-Induced Genes and Leads to the Hyperactivation of WIPK.

(A) and (B) Extended ozone exposure reveals strong and prolonged activation of WIPK in the RI line. A temporal profile of the phosphorylation status of SIPK and WIPK was generated through anti-pERK immunoblotting of crude proteins extracted from tissues of either wild-type and RI lines (A) or OX and AEF lines (B) exposed to ozone for different times (0 to 8 h).

(C) Alteration of SIPK does not lead to changes in the amount of WIPK. Protein extracts from untreated and 4-h ozone-treated tissues from different kinotypes were immunoblotted with anti-WIPK antibody. WT, wild type.

et al., 1999) in transgenic tobacco plants. On the other hand, ozone-induced expression of GST, a general cellular protectant, was suppressed strongly in the OX line, but its expression was delayed markedly in the RI line. In Arabidopsis, both hydrogen peroxide and ozone induce GST expression (Clayton et al., 1999; Grant et al., 2000), and this expression has been demonstrated to require the activity of an unidentified 48-kD MAPK and calcium ion influx. Calcium channel activity also is essential for the ROS activation of SIPK in tobacco (Samuel et al., 2000).

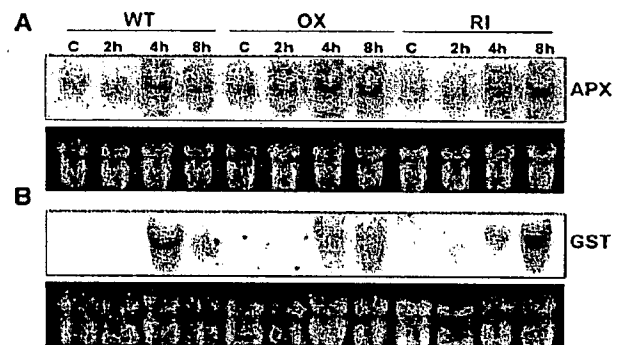
The delayed response of the antioxidant genes in the RI line could result in increased early accumulation of ROS (Figure 4B), which could lead to a necrotic cell death process. In the OX line, although the cAPX gene response to ozone appeared to be normal, the antioxidant response clearly was unable to contain the increasing ROS levels associated with extended SIPK activation (Figure 4B). MAPK activation has been linked previously to increased ROS accumulation in Arabidopsis (Ren et al., 2002). A broader comparison of transcript profiles should generate useful insights into other connections between the transmission of redox signals by SIPK and the ability of the cell to avoid oxidative cell death.

Another aspect of the link between SIPK activation and cell death is revealed in the pattern of MAPK activation in ROS-stressed plants. The activation of SIPK by ozone occurred within 10 min in SIPK-OX plants but was not reversed for 4 h, by which time cell death already was becoming visible. This outcome is similar to the association of the prolonged activa-

tion of mammalian ERK with the induction of programmed cell death in neurons (Stanciu et al., 2000). Considered together with the results of the transient expression experiments, this finding demonstrates that the unregulated continuous activity of SIPK within plant cells profoundly affects normal homeostatic mechanisms.

The absence of SIPK in the SIPK-RI genotype also led to premature cell death under redox stress conditions, but in this case, the hyperactivated species observed was WIPK rather than SIPK. There have been other indications that WIPK plays a central role in plant stress signaling. This gene was identified originally on the basis of its rapid and transient induction upon wounding of tobacco leaves (Seo et al., 1995), and the gene product was shown later to be activated transiently by wounding (Seo et al., 1999) and by various other stresses (Romeis et al., 1999; Zhang et al., 2000). WIPK activation usually is accompanied by the activation of SIPK, but SIPK and WIPK do not always respond in unison; some oxidative stresses appear to activate SIPK preferentially and leave WIPK unaffected (Kumar and Klessig, 2000; Samuel et al., 2000).

WIPK activity, either alone or together with SIPK, has been suggested to be involved in the induction of cell death in cultured tobacco cells by specific fungal elicitor treatments (Zhang et al., 2000). Pretreatment of the elicited cells with staurosporine and K252A (protein kinase inhibitors) completely suppressed both WIPK activation and cell death. However, transient overexpression of WIPK did not result in its activation and failed to induce cell death in infiltrated tobacco leaves, unlike overexpression of SIPK (Zhang and Liu, 2001). In another study, the stable overexpression of WIPK in transgenic tobacco was accompanied by the constitutive expression of protease inhibitor II and the accumulation of methyl jasmonate



**Figure 8.** Alteration of SIPK Signaling Affects the Expression of GST and cAPX.

RNA gel blot analysis of the accumulation of cAPX (A) and GST mRNA (B) in wild-type, SIPK-overexpressing, and SIPK-suppressed transgenic tobacco. Plants were exposed to ambient air (C) or 500 ppb of ozone for 2, 4, and 8 h, and total RNA was harvested from the third and fourth leaves.

WT, wild type.

(Seo et al., 1999), but the oxidative stress sensitivity of the WIPK-OX lines was not reported.

How SIPK elimination leads to the prolonged hyperactivation of WIPK is unknown, but various possibilities suggest themselves. If NtMEK2 is the sole upstream MAPK kinase responsible for the activation of both SIPK and WIPK, these two MAPKs may normally compete for binding to NtMEK2. However, basal levels of SIPK in unstimulated tobacco cells are much higher (10-fold) than those of WIPK (Zhang and Klessig, 1998b). In the absence of competition from SIPK, activation of WIPK by NtMEK2 activation in SIPK-RI cells may be much more efficient than usual. This scenario also might explain why WIPK remains largely inactivated in ozone-treated SIPK-OX tissues in which an excess of SIPK is present. However, although this model accounts for WIPK hyperactivation, it does not necessarily explain why that activation is prolonged abnormally.

Alternatively, one of the normal roles of activated SIPK may be the direct or indirect regulation of WIPK activity. Both dual-specificity phosphoprotein phosphatases (MKP) and Ser/Thr phosphatases have been implicated in inactivating MAPK pathways in mammalian and plant models (Brondello et al., 1997; Meskiene et al., 1998; Ulm et al., 2001; Westermarck et al., 2001). If SIPK activity is required for the induction or activation of a protein phosphatase that normally acts upon phospho-WIPK, the absence of SIPK from oxidant-stressed SIPK-RI cells would create a situation in which WIPK could be activated by its cognate MAPK kinase but could not be inactivated subsequently.

In this regard, it is interesting that Arabidopsis plants in which a dual-specificity phosphatase (AtMKP-1) has been mutated by T-DNA insertional mutagenesis display increased activation of an unidentified ~49-kD MAPK and are more susceptible to ROS-generating stresses (e.g., UV light) (Ulm et al., 2001). On the other hand, MP2C, an alfalfa Ser/Thr phosphatase belonging to the PP2C class, has been shown to be a negative regulator of the MAPK pathway involving stress-activated MAPK, an apparent ortholog of WIPK (Meskiene et al., 1998). Both classes of protein phosphatase could be involved in cross-regulation mechanisms. Resolution of this question, and of the relative importance of the loss of SIPK activity versus the enhancement of WIPK activity in controlling oxidant-induced cell death, will require the development and analysis of other relevant single and multiple loss-of-function genotypes. These studies are now under way.

## METHODS

### Plant Material and Treatment

Tobacco (*Nicotiana tabacum*) plants of all genotypes were grown for 6 weeks in soil under controlled environmental conditions (25/20°C, 16-h-light/8-h-dark cycle) and then exposed to ozone (500 parts per billion) and harvested as described previously (Orvar and Ellis, 1997).

### Recombinant Protein Production

The open reading frame (ORF) of salicylate-induced protein kinase (SIPK) was amplified by reverse transcriptase-mediated (RT) PCR using gene-specific primers and RNA isolated from untreated leaves of tobacco cv Xanthi-nc. The amplicon was cloned in frame into the expression vector pGEX 4T-3. Mutations in the activation loop of SIPK were introduced using a PCR-mediated approach, taking advantage of the unique NheI restriction site close to the activation loop. The mutational primers were designed so that the mutant form would code for AEF instead of TEY at amino acid positions Thr-218 and Tyr-220.

The SIPK(AEF) gene construct then was cloned into pGEX 4T-3. The recombinant glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 cells by induction with 0.1 mM isopropylthio- $\beta$ -galactoside for 4 h at 25°C, followed by purification according to the manufacturer's protocol (Amersham Pharmacia). The different constructs were sequenced to confirm the changes and the absence of mismatches.

### Intron-Spliced Hairpin Loop RNA-SIPK Construct

The double-stranded RNA interference construct was tailored through a PCR-mediated approach using the N-terminal sequence of the SIPK ORF. A minimal intron based on the splice junctions and flanking regions of the fourth intron of *AtMPK6* (the Arabidopsis ortholog of SIPK) was incorporated into the sense-strand primer. The sense strand then was amplified using a primer combination that generated an EcoRI cleavage site and intron-XbaI sequence on the opposite ends of the product, whereas the antisense strand was amplified using a primer combination that added BamHI and XbaI sites on the opposite ends of the product. These two products were directionally cloned into EcoRI-BamHI-processed Bin19/pRT101 through a triple ligation, which placed the RNA interference construct under the control of the 35S promoter of *Cauliflower mosaic virus* (Figure 3A).

### Binary Vector Construction and Plant Transformation

The different SIPK overexpression constructs were tagged with a C-terminal FLAG epitope through a PCR-mediated approach, followed by ligation into the plant expression vector Bin19/pRT101, which contains an *nptII*-selectable marker. All of the constructs were sequenced to confirm the presence of appropriate changes. The recombinant binary vector was used to transform competent *Agrobacterium tumefaciens* (EHA105) cells by a freeze-thaw transformation procedure.

*Agrobacterium*-mediated transformation of tobacco (cv Xanthi-nc) was performed using a leaf disc cocultivation procedure. Transformants were selected on half-strength Murashige and Skoog (1962) culture medium containing 50 mg/L kanamycin. Surviving plantlets were screened by PCR using 35S forward and gene-specific reverse primer combinations. Positive transformants then were screened by protein gel blot analysis (see below) using an anti-FLAG antibody for the SIPK overexpression lines and anti-SIPK antibodies to assess the RI suppression lines.

The confirmed transgenic lines were transferred to soil and grown to maturity, and seeds were collected. The T1 seeds were germinated on half-strength Murashige and Skoog (1962) medium with 50 mg/L kanamycin, and antibiotic-resistant plants were transferred to soil and grown under controlled conditions.

### Transient Transformation Using *Agrobacterium* Infiltration

Four- to 6-week-old wild-type tobacco plants (cv Xanthi-nc) were used for infiltration experiments as described previously (Yang et al., 2001). This involved leaf infiltration with a mixed culture (OD of 0.4 at 600 nm) of *Agrobacterium* EHA105 containing the SIPK-FLAG overexpression construct plus an equal population of *Agrobacterium* containing either the empty vector or the SIPK-Rl construct. At the indicated times (Figure 1), the infiltrated area was cut from the leaf, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until further analysis.

### RNA Gel Blot and RT-PCR Analysis

Total RNA (15  $\mu\text{g}$ ) was resolved on 1% agarose-formaldehyde gels, blotted, and probed as described previously (Orvar and Ellis, 1997). The 600-bp C-terminal fragment of the *SIPK* ORF and PCR-amplified fragments of the cytosolic ascorbate peroxidase (*cAPX*) and *GST* were used as probes. Gene-specific primers were used to amplify the ORFs of *cAPX* (forward, 5'-AGAACAATTGCTATGGGTAAGTG-3'; reverse, 5'-GCAAGCTTAAGCTTCAGCAAAT-3') and *GST* (forward, 5'-ATGGCGATCAAAGTCCATGGTA-3'; reverse, 5'-TTTTTCAGCTTCTCCAATCCC-3') using cDNA as the template.

The cDNA was synthesized from total RNA extracted from control and ozone-exposed tissues of the different genotypes/treatments using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). RT-PCR was performed using gene-specific primers designed to target either *SIPK* (25 cycles) or *NTF4* (30 cycles). The number of cycles was adjusted so that the amplification was within the linear range. As an internal control, 18S ribosomal cDNA was amplified using a 1:4 ratio of 18S-specific primers to competitor's DNA fragments provided by Ambion (Austin, TX).

### Protein Extraction and Protein Gel Blot Analysis

Total protein extracts were prepared (40 to 80  $\mu\text{g}$ ) and used for protein gel blot analysis as described previously (Samuel et al., 2000). A primary antibody dilution of 1:1000 was used for anti-pERK (New England Biolabs, Beverly, MA), and a dilution of 1:5000 was used for anti-SIPK, anti-wound-induced protein kinase (WIPK) (Seo et al., 1999; Y. Ohashi, personal communication), and anti-FLAG (Sigma) antibodies.

### Immune Complex Kinase Assay

Immunoprecipitations were performed as described previously (Samuel et al., 2000) using 250  $\mu\text{g}$  of extracted protein together with 5  $\mu\text{g}$  of either anti-SIPK or anti-WIPK antibodies. The immunoprecipitates were analyzed in an in gel kinase assay as described previously using myelin basic protein as the substrate (Zhang and Klessig, 1997).

### In Vitro Kinase Assays

GST fusion proteins (5  $\mu\text{g}$ ) of the wild-type and mutant SIPK(AEF) were incubated with 5  $\mu\text{g}$  of myelin basic protein and 10  $\mu\text{Ci}$  of  $\gamma\text{-}^{32}\text{P}$ -labeled ATP ( $>5000\text{ Ci/mmol}$ ) (Amersham Pharmacia) in a 20- $\mu\text{L}$  reaction mixture (20 mM Hepes, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 5 mM  $\beta$ -mercaptoethanol, 2 mM  $\text{Na}_2\text{VO}_4$ , and 20 mM  $\beta$ -glycerophos-

phate) at  $30^{\circ}\text{C}$  for 30 min. The reaction was stopped with 6  $\times$  SDS loading buffer, and the samples were resolved on a 15% polyacrylamide gel, blotted onto a nylon membrane, and visualized by autoradiography.

### Ion-Leakage Assay

Five leaf discs (9 mm) were cut from each of the third and fourth leaves of ozone-exposed and untreated plants of the wild type, OX4, and R15 lines. The 10 leaf discs were incubated in 5 mL of deionized water at  $25^{\circ}\text{C}$  on a gyratory shaker at 110 rpm for 4 h, and the conductivity of the solution was measured as described previously (Mittler et al., 1999).

### In Situ Staining for Hydrogen Peroxide

Hydrogen peroxide was visualized in situ by 3,3'-diaminobenzidine staining performed essentially according to Torres et al. (2002). Leaf halves were collected after 8 h of ozone exposure (500 parts per billion) and vacuum infiltrated with the 3,3'-diaminobenzidine (1 mg/mL) solution. Infiltrated leaves were placed under high humidity until brown precipitation was observed (5 to 6 h) and then fixed with a solution of ethanol:lactic acid:glycerol (3:1:1, v/v) for 2 days, followed by further clearing in methanol. Unless indicated otherwise, all experiments were repeated with consistent results.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

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# A Classical Arabinogalactan Protein Is Essential for the Initiation of Female Gametogenesis in Arabidopsis

Gerardo Acosta-García and Jean-Philippe Vielle-Calzada<sup>1</sup>

Laboratory of Reproductive Development and Apomixis, Department of Genetic Engineering, Centro de Investigación y Estudios Avanzados, Unidad Irapuato, Irapuato Guanajuato, Mexico

Classical arabinogalactan proteins (AGPs) are an abundant class of cell surface proteoglycans widely distributed in flowering plants. We have used a combination of enhancer detection tagging and RNA interference (RNAi)-induced posttranscriptional silencing to demonstrate that *AGP18*, a gene encoding a classical arabinogalactan protein, is essential for female gametogenesis in *Arabidopsis thaliana*. *AGP18* is expressed in cells that spatially and temporally define the sporophytic to gametophytic transition and during early stages of seed development. More than 75% of the T1 transformants resulted in T2 lines showing reduced seed set during at least three consecutive generations but no additional developmental defects. *AGP18*-silenced T2 lines showed reduced *AGP18* transcript levels in female reproductive organs, the presence of 21-bp RNA fragments specific to the *AGP18* gene, and the absence of in situ *AGP18* mRNA localization in developing ovules. Reciprocal crosses to wild-type plants indicate that the defect is female specific. The genetic and molecular analysis of *AGP18*-silenced plants containing a single T-DNA RNAi insertion suggests that posttranscriptional silencing of *AGP18* is acting both at the sporophytic and gametophytic levels. A cytological analysis of all defective *AGP18*-RNAi lines, combined with the analysis of molecular markers acting at key stages of female gametogenesis, showed that the functional megaspore fails to enlarge and mitotically divide, indicating that *AGP18* is essential to initiate female gametogenesis in Arabidopsis. Our results assign a specific function in plant development to a gene encoding a classical AGP.

## INTRODUCTION

The life cycle of flowering plants consists of a diploid sporophytic phase and two morphologically different haploid gametophytic phases taking place in specialized reproductive organs. Distinct types of meiotically derived cells give rise to the male and female gametophytic phases. In the anther, many microsporocytes develop into pollen grains, which harbor the sperm cells and represent the male gametophyte. In the ovule, usually a single sporophytic cell (the megaspore mother cell [MMC]) undergoes meiosis and gives rise to four haploid products (the megaspores) during a process referred to as megasporogenesis. While three of the megaspores undergo programmed cell death, a single functional megaspore enlarges and gives rise to the female gametophyte (or megagametophyte). In *Arabidopsis thaliana*, female gametogenesis initiates when the single functional megaspore divides mitotically to form an eight-nucleate syncytium. Subsequent cellularization partitions the eight nuclei into seven cells: an egg cell and two synergids at the distal (or micropylar) pole of the female gametophyte, three antipodals at the proximal (or chalazal) pole, and a binucleated central cell whose nuclei

fuse before fertilization. This type of development and organization of the female gametophyte defines the Polygonum type that prevails in >70% of the species examined (Maheshwari, 1950; Willemse and van Went, 1984; Reiser and Fischer, 1993; Drews and Yadegari, 2002). Whereas the fusion of a sperm with the egg cell forms a zygote that subsequently develops into an embryo, fertilization of the binucleated central cell eventually gives rise to the endosperm, a triploid tissue essential for seed viability.

Little is known about the genetic basis and molecular mechanisms that regulate the initiation of female gametogenesis in the ovule. A large collection of both sporophytic and gametophytic mutants defective in female gametophyte development has been identified in Arabidopsis (Schneitz et al., 1997; Christensen et al., 1998; Howden et al., 1998; Grini et al., 1999; Drews and Yadegari, 2002). Whereas sporophytic mutations act at the diploid level and are inherited in a Mendelian 3:1 ratio, gametophytic mutants are poorly transmitted through either one or both types of gametes and exhibit distorted segregation patterns. Many mutants that disrupt meiosis have been isolated (Klimyuk and Jones, 1997; Siddiqi et al., 2000; Yang and Sundaresan, 2000; Reddy et al., 2003), but little is known about other developmental aspects of megasporogenesis. To date, *SPOROCTELESS (SPO)/NOZZLE* is the only gene shown to be required for the initiation of microsporogenesis and megasporogenesis (Schieffthaler et al., 1999; Yang et al., 1999). *SPO* encodes a nuclear protein related to MADS box transcription factors that is expressed during early anther and ovule development. In plants homozygous for *hadad (hdd)* and *proliferá (prf)*, female gametophyte development is arrested at either the two-nucleate or the four-nucleate stage, respectively. Whereas the

<sup>1</sup>To whom correspondence should be addressed. E-mail vielle@ira.cinvestav.mx; fax 52-462-624-58-49.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jean-Philippe Vielle-Calzada (vielle@ira.cinvestav.mx).

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gene responsible for the mutation in *hdd* has yet to be identified (Moore et al., 1997), *PRL* encodes a Mcm7-like licensing factor essential for DNA replication (Springer et al., 1995). Two insertional alleles in *CYTOKININ-INDEPENDENT 1*, a gene encoding a putative Arabidopsis His kinase, were shown to cause nuclear degeneration in the female gametophyte as early as the four-nucleate stage (Christensen et al., 1998; Pischke et al., 2002). Recently, the *NOMEGA* gene was shown to be required for cell cycle progression beyond the two-nucleate stage; *NOMEGA* encodes a putative APC6/CDC16 component of the anaphase promoting complex in Arabidopsis (Kwee and Sundaresan, 2003). Several additional gametophytic mutants that fail to progress beyond the one-nucleate haploid stage have been identified (Christensen et al., 1998), but the corresponding genes have yet to be isolated and characterized.

Numerous studies showing that modifications of plant growth conditions can alter the sporophyte to gametophyte transition (Bell, 1989) or even the whole plant reproductive outcome (Knox, 1967) indicate that the presence of molecular signals that determine the fate of competent cells is fundamental for switching from a sporophytic into a gametophytic developmental pathway. Several regulatory proteins have been shown to have important functions in cell signaling and recognition during plant development. Arabinogalactan proteins (AGPs) are an abundant and heterogeneous class of highly glycosylated Hyp-rich glycoproteins widely distributed in the plant kingdom (Fincher et al., 1974; Clarke et al., 1979; Kreuger and van Holst, 1996; Sommer-Knudsen et al., 1998; Gaspar et al., 2001; Showalter, 2001). The recent characterization of genes encoding different AGP backbones in several species gave rise to the current distinction between classical and nonclassical AGPs (Chen et al., 1994; Du et al., 1994; Mau et al., 1995; Knox, 1999). Classical AGPs contain a domain responsible for attaching the protein backbone to a glycosylphosphatidylinositol (GPI) membrane anchor (Schultz et al., 1998; Youl et al., 1998; Sherrier et al., 1999; Schindelman et al., 2001; Bornier et al., 2002; Sun et al., 2004). By contrast, nonclassical AGPs lack the GPI anchor signal and are soluble components of the extracellular matrix often containing Asn- or Hyp-rich domains (Majewska-Sawka and Nothnagel, 2000; Schultz et al., 2000; Gaspar et al., 2001). Molecular and biochemical evidence indicates that AGPs have specific functions during root formation (Willats and Knox, 1996; Casero et al., 1998; van Hengel and Roberts, 2002), the promotion of somatic embryogenesis (Serpe and Nothnagel, 1994; Kreuger and van Holst, 1993, 1996; van Hengel et al., 2001; van Hengel and Roberts, 2002), or the attraction of pollen tubes in the style (Du et al., 1994; Cheung et al., 1995; Wu et al., 1995; Jauh and Lord, 1996; Roy et al., 1998). The use of monoclonal antibodies directed against carbohydrate epitopes provided evidence suggesting that AGPs play an important role during the alternation between sporophytic and gametophytic transitions in the ovule (Pennell and Roberts, 1990; Pennell et al., 1991; McCabe et al., 1997). Although these studies elegantly showed that the establishment of a female reproductive lineage is associated with changes in the distribution of AGP epitopes (Pennell et al., 1992), they did not identify a specific AGP protein or the corresponding gene acting during ovule development or early embryo formation. A few mutations altering the activity of

specific genes encoding AGPs in Arabidopsis have been described. Homozygous plants for *resistant to agrobacterium transformation 1* are resistant to root-dependent transformation via *Agrobacterium tumefaciens* (Nam et al., 1999); however, mutant plants are phenotypically indistinguishable from the wild type, and no developmental defects associated with the mutation have been described. The mutation is caused by a T-DNA insertion within the promoter region of the Arabidopsis *AGP17* gene (Gaspar et al., 2001). A second insertional mutant in a gene encoding a nonclassical AGP (*AGP30*) has recently been shown to be involved in root regeneration and seed germination (van Hengel and Roberts, 2003). Recently, hybrid-type proteoglycans having properties of both AGPs and lipid-transfer proteins have been shown to be essential for the differentiation of tracheary elements in *Zinnia elegans* and Arabidopsis (Motosé et al., 2004).

In this study, we report the enhancer detection-based identification of *AGP18*, a classical AGP gene that specifically acts during female gametophyte development in Arabidopsis. To determine the function of *AGP18*, we introduced double-stranded RNA in wild-type plants and specifically degraded the endogenous *AGP18* transcript by RNA interference (RNAi). More than 75% of the primary transformants resulted in lines showing reduced seed set but no additional developmental abnormalities. Reciprocal crosses to wild-type plants suggested that the defect is female specific. The genetic and molecular analysis of a line containing a single T-DNA RNAi insertion suggests that posttranscriptional silencing of *AGP18* is acting both at the sporophytic and gametophytic levels. The cytological analysis of all defective *AGP18*-RNAi lines indicates that *AGP18* is essential to initiate female gametogenesis in Arabidopsis. Our results assign a specific function in plant development to a gene encoding a classical AGP.

## RESULTS

### Enhancer Detection Tagging of *AGP18*

Using the system established by Sundaresan et al. (1995), we have generated a *Ds* enhancer detector and a gene trap population to identify patterns of expression associated with genes acting during female gametophyte development in Arabidopsis. The enhancer detection vector relies on a maize (*Zea mays*) *Ds* transposon carrying a  $\beta$ -glucuronidase reporter gene (*uidA* or *GUS*) under the control of a minimal promoter. Such a reporter construct is not trapping genes but rather integrating into genomic sequences to serve as a detector of any given regulatory sequence that is acting as an enhancer of promoter activity at the specific location of the insertion (Bellen, 1999; Springer, 2000). The *Ds* enhancer detector element (DsE) also contains the neomycin phosphotransferase II (*NPTII*) gene (conferring resistance to kanamycin); *NPTII* acts as a selectable marker and facilitates the genetic analysis of segregating enhancer detector or gene trap lines.

Whole-mount staining and clearing procedures allow screening for reporter gene expression (*GUS*) at different developmental stages encompassing megasporogenesis and female gametogenesis, from the time when the ovule primordium has just started its elongation (before MMC differentiation) to stages

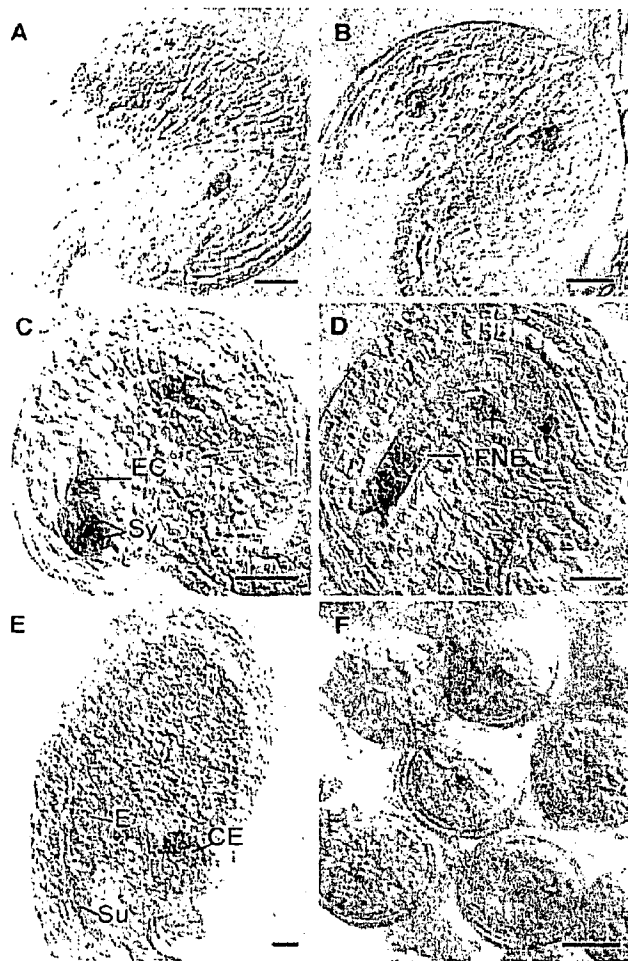
where the female gametophyte is fully differentiated (J.-P. Vielle-Calzada and U. Grossniklaus, unpublished results; Vielle-Calzada et al., 2000). Figure 1 shows the pattern of GUS expression identified in MET333. MET333 shows initial GUS expression in the chalazal region of the four-nucleate female gametophyte (Figure 1A). At the eight-nucleate stage, expres-

sion is restricted to the young antipodal cells and the cellularizing egg apparatus (Figure 1B). At maturity, the female gametophyte shows GUS expression in the synergids, the egg cell, and the antipodals but not in the central cell (Figure 1C). This pattern of expression persists after fertilization (Figure 1D); however, GUS is also expressed in the free nuclear endosperm after fertilization of the central cell (Figure 1D). During early seed development, GUS is expressed in the embryo proper, the suspensor, and the chalazal endosperm (Figure 1E). Interestingly, MET333 also shows GUS expression in cytoplasmic domains closely associated with the vegetative nucleus of mature pollen grains and in pollen tubes (Figure 1F) but not in microsporocytes at earlier stages of development. A detailed analysis of MET333 plants homozygous for kanamycin resistance did not reveal a mutant phenotype at any stage of plant reproductive development.

DNA gel blot analysis using a DsE-specific probe that includes a portion of *NPTII* showed that two copies of the DsE element were present in MET333 heterozygotes but were absent from wild-type siblings in which GUS expression was not detected (data not shown). Genomic sequences flanking both DsE elements were rescued using thermal asymmetric interlaced PCR (Liu et al., 1995). Sequence analysis of the PCR products showed that the DsE elements were inserted 254 and 995 bp upstream of the transcription initiation site of *AGP18* (At4g37450). Figure 2A illustrates the molecular structure of *AGP18* and the localization of both DsE insertion sites. *AGP18* encodes a classical AGP containing a C-terminal domain responsible for anchoring the protein to GPI. In animals, GPI anchors have been shown to provide an alternative to transmembrane proteins for anchoring proteins to components of the cell surface (Takos et al., 1997, 2000; Schultz et al., 1998; Svetek et al., 1999; Borner et al., 2002; Sharma et al., 2004). Additionally, *AGP18* contains an N-terminal secretory signal predicted to direct the secretion of the protein via the endoplasmic reticulum, two Pro-rich domains that are possible targets of glycosylation (Tan et al., 2003), and a Lys-rich domain predicted to interact with negatively charged molecules (Figure 2B; Gilson et al., 2001; Schultz et al., 2002). The Lys-rich domain is present in only 3 of 15 classical AGPs found to be encoded in the genome of Arabidopsis (Schultz et al., 2002). RT-PCR analysis revealed that the levels of *AGP18* transcription in MET333 are similar to the wild type, confirming that *AGP18* expression is not diminished by the presence of the DsE insertions (Figure 2C).

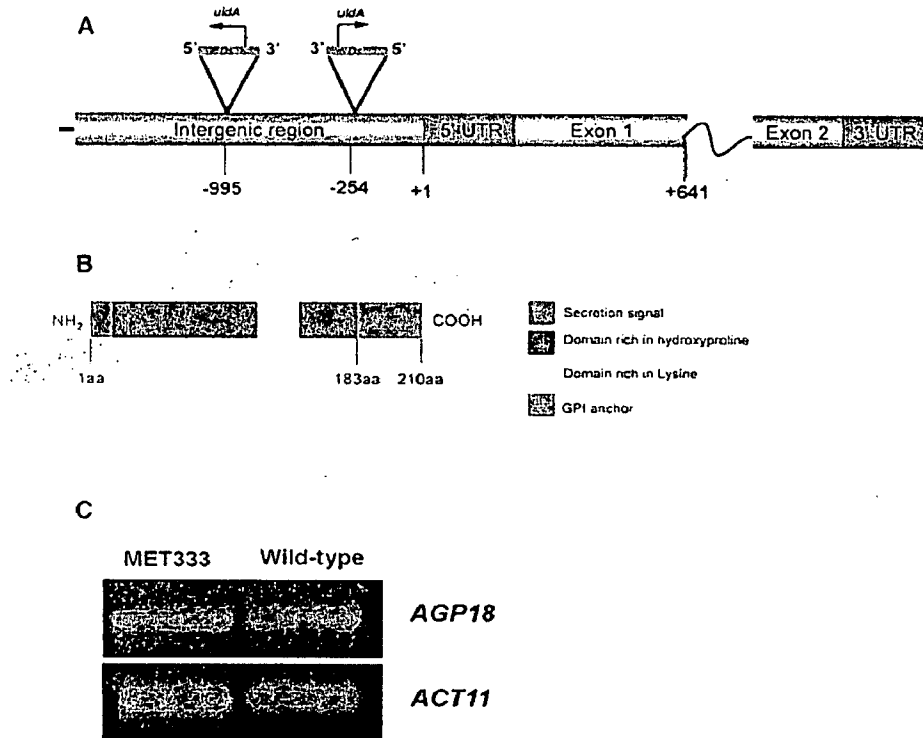
#### ***AGP18* Is Expressed in Adjacent Sporophytic and Gametophytic Cells**

To determine if the pattern of GUS expression identified in MET333 reflected the pattern of expression of *AGP18*, we determined the localization of *AGP18* mRNA by in situ hybridization. To avoid the detection of mRNA corresponding to other AGP transcripts structurally resembling *AGP18*, sense and antisense digoxigenin-labeled probes were generated using a specific portion of the first exon that shows no homology with other AGP genes. The results are summarized in Figure 3. Detailed analysis of all aerial parts of Arabidopsis demonstrated that *AGP18* mRNA could be localized only in developing anthers and ovules and transiently in clusters of companion cells closely associated with vascular elements of the stem. *AGP18* is initially



**Figure 1.** Pattern of GUS Expression in the Enhancer Detector Line MET333.

(A) Female gametophyte at four-nucleate stage.  
(B) Cellularized female gametophyte.  
(C) Mature female gametophyte before fertilization.  
(D) Female gametophyte after fertilization.  
(E) Embryo at four-cell stage.  
(F) Mature pollen with GUS expression associated with the vegetative nucleus.  
Sy, synergids; EC, egg cell; E, embryo; FNE, free nuclear endosperm; Su, suspensor; CE, chalazal endosperm. Bars in (A) to (E) = 20  $\mu$ m; bar in (F) = 10  $\mu$ m.



**Figure 2.** Genomic Structure and Protein Organization of AGP18.

The enhancer detector line MET333 has two DsE elements inserted in the 5' regulatory region of *AGP18*.

(A) Genomic structure of *AGP18*. The arrows show the direction of transcription of *uidA* (GUS).

(B) Predicted protein structure of AGP18. aa, amino acids.

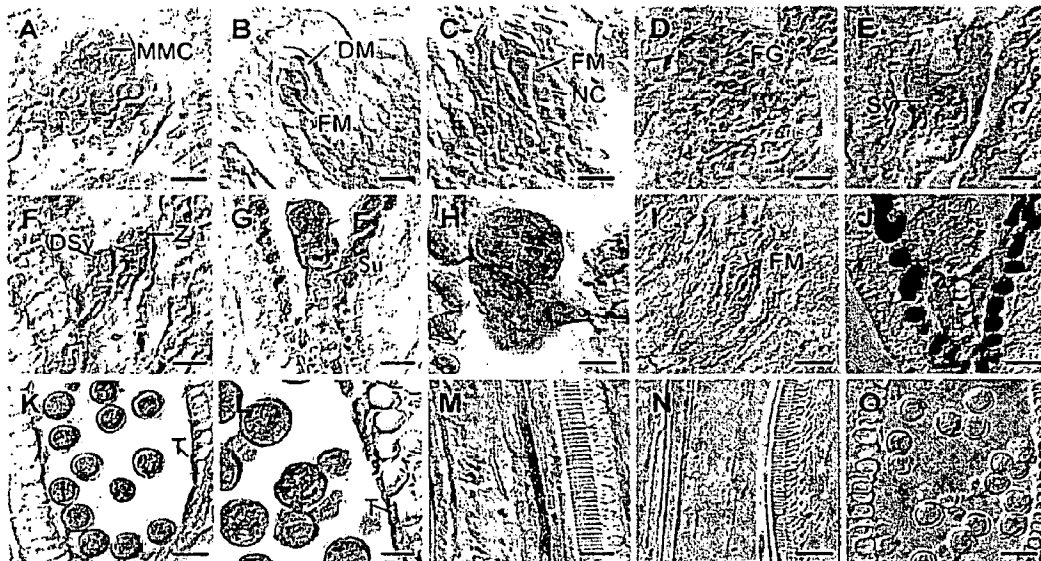
(C) RT-PCR analysis shows that the levels of transcription of *AGP18* are identical in MET333 and wild-type plants.

expressed in the MMC and the neighboring nucellar cells of the young ovule primordium (Figure 3A). *AGP18* expression persists in all four products of female meiosis (Figure 3B). At the end of megasporogenesis, when the three nonfunctional megaspores have already degenerated, *AGP18* mRNA is abundant in the functional megaspore but also in the adjacent nucellar cells (Figure 3C). During female gametogenesis, *AGP18* is expressed in the developing female gametophyte (Figure 3D). At maturity, abundant *AGP18* mRNA can be detected in the synergids (Figure 3E), the egg cell, and the antipodals but not in the central cell. After fertilization, *AGP18* mRNA is present in the developing embryo as well as in the free nuclear endosperm (Figures 3F to 3H). Abundant levels of *AGP18* mRNA persist in the embryo until the late globular stage and subsequently start to decrease. No *AGP18* mRNA can be detected in seeds containing torpedo or cotyledonary embryos. In the anther, *AGP18* is expressed in the tapetum and the mature pollen grain (Figures 3K and 3L). These results indicate that GUS expression in MET333 overlaps with the localization of *AGP18* mRNA, confirming that DsE elements partially detect the expression of *AGP18*; however, the absence of GUS expression in MET333 sporophytic cells (the developing nucellus and the mature tapetum) suggests that additional

regulatory elements driving the expression of *AGP18* are not detected by either DsE element.

#### Generation of *AGP18*-RNAi Plants and Analysis of RNA Levels

To determine the role of *AGP18* in Arabidopsis, a 740-bp fragment of the *AGP18* cDNA was cloned into a pFGC5941 RNAi vector (Kerschen et al., 2004) in both sense and antisense orientations and used to transform wild-type Columbia plants. Figure 4A illustrates the RNAi construct that was used to conduct these experiments. pFGC5941 contains a 35S promoter of *Cauliflower mosaic virus* (CaMV35S) that drives the transcription of a partial *AGP18* sequence cloned in both sense and antisense orientations and separated by an intron of the chalcone synthase gene. After formation of hairpin RNA structures, the resulting double-stranded RNA transcripts can cause posttranscriptional silencing of endogenous gene activity (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000; Smith et al., 2000). Although a detailed pattern of CaMV35S promoter activity during male and female gametogenesis has yet to be determined in Arabidopsis, we reasoned that *AGP18* transcripts localized in sporophytic



**Figure 3.** Localization of *AGP18* mRNA by in situ hybridization.

(A) MMC stage. Bar = 9  $\mu$ m.

(B) Female meiosis stage. Bar = 8  $\mu$ m.

(C) Functional megaspore stage with young nucellus. Bar = 8.5  $\mu$ m.

(D) Two-nucleate stage female gametophyte. Bar = 14  $\mu$ m.

(E) Mature female gametophyte. Bar = 8  $\mu$ m.

(F) Zygote stage. Bar = 9  $\mu$ m.

(G) Embryo four-cell stage. Bar = 15  $\mu$ m.

(H) Embryo at early globular stage. Bar = 11  $\mu$ m.

(I) Functional megaspore and young nucellus, sense probe. Bar = 15  $\mu$ m.

(J) Embryo at early globular stage, sense probe. Bar = 20  $\mu$ m.

(K) Mature pollen. Bar = 24  $\mu$ m.

(L) Anther showing the tapetum. Bar = 20  $\mu$ m.

(M) Longitudinal section of a stem. Bar = 20  $\mu$ m.

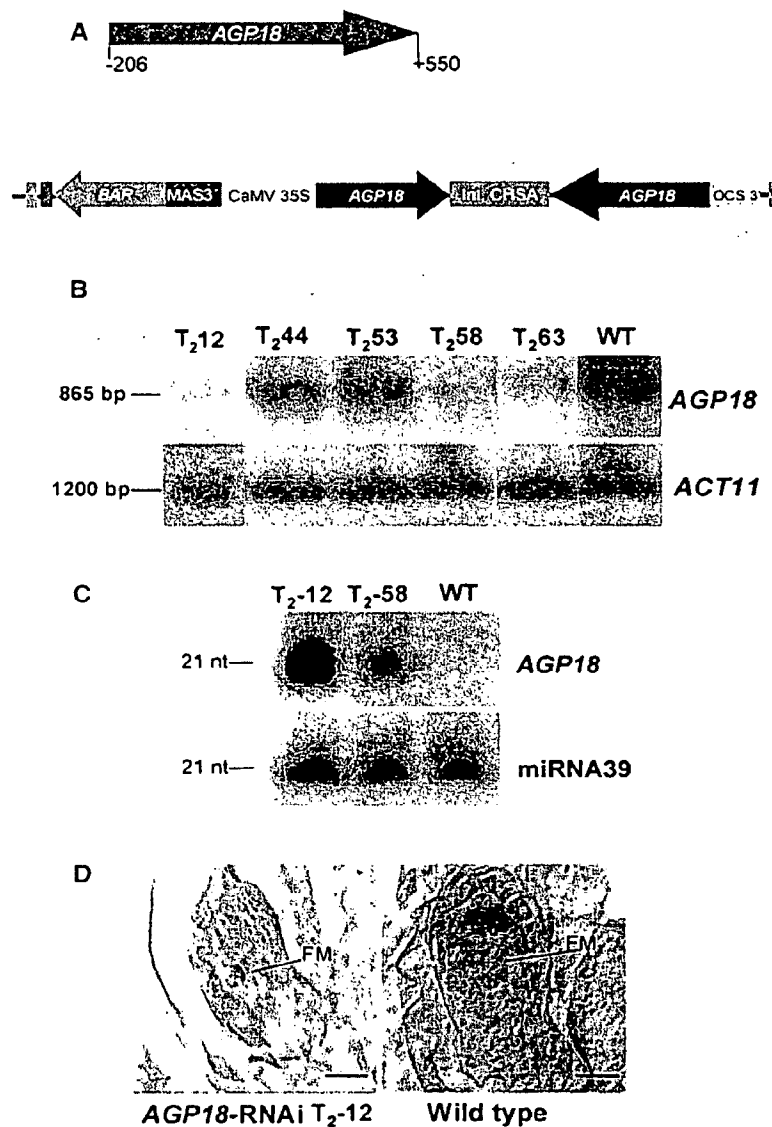
(N) Longitudinal section of a stem, sense probe. Bar = 20  $\mu$ m.

(O) Mature pollen, sense probe. Bar = 20  $\mu$ m.

(A) to (H) and (K) to (M) hybridizations with antisense probe; (I), (J), (N), and (O) hybridizations with sense probe. NC, nucellar cells; FM, functional megaspore; DM, degenerating megaspores; Sy, synergids; DSV, degenerating synergid; FG, female gametophyte; Z, zygote; E, embryo; Su, suspensor; T, tapetum.

cells can be the target of RNAi-dependent silencing driven by CaMV35S. After floral-dipping transformation, 75 primary transformants were generated, none of which showed visible defects during vegetative growth, root development, or floral organogenesis; however, 58 out of 75 adult T1 transformants showed semisterility defects. All 58 transformants maintained a reduced fertility phenotype in the T2 generation. To determine a possible relationship between a decrease in *AGP18* transcript levels and the defective phenotype, RNA was extracted from developing gynoecia of *BASTA*-resistant *AGP18*-RNAi T2 lines and used for RNA gel blot analysis. The results are shown in Figure 4B, with actin as a constitutive control to show that equal amounts of RNA were used. Compared with wild-type plants, all 10 T2 lines tested showed a substantial decrease in the transcripts levels of *AGP18*. Among those lines, T2-12, T2-44, and T2-58 showed significantly >50% reduction in seed set, whereas

T2-63 and T2-53 had a 27.4 and 49.8% reduction, respectively. No correlation was found between the level of *AGP18* expression determined by RNA gel blot analysis and the degree of sterility; this absence of correlation has been documented in previous studies showing that abnormal phenotypes induced by RNAi are not always associated with a detectable decrease in transcript levels (Kerschen et al., 2004; C. Napoly and R. Jorgensen, personal communication). The induction of posttranscriptional gene silencing has been shown to result in the production of 21- to 23-bp RNA fragments with a sequence identical to a portion of the silenced gene (Elbashir et al., 2000). To determine if the production of 21- to 23-bp RNA fragments could be associated with the degradation of the *AGP18* transcript, polyacrylamide gels were used to detect the presence of small RNA fragments corresponding to *AGP18*. As shown in Figure 4C, small RNAs corresponding to *AGP18* were detected in lines in which



**Figure 4.** Accumulation of *AGP18* Transcript, Presence of 21-bp Small RNAs, and Absence of *AGP18* Expression in the Gynoecium of *AGP18*-RNAi T2 Lines.

(A) Schematic diagram of the vector used to posttranscriptionally silence *AGP18*. The arrow indicates the sequence cloned in the RNAi silencing vector. Numbers indicate nucleotide positions with respect to initiation of the *AGP18* mRNA.

(B) Expression analysis of four *AGP18*-RNAi T2 lines and a wild-type control. RNA was isolated from mature gynoecia in both silenced and wild-type plants. A portion of the *AGP18* cDNA was used as a probe. RNA gel blots were subsequently rehybridized with a specific actin probe (*ACT11*) as a loading control.

(C) A polyacrylamide gel of 100  $\mu$ g of low molecular weight RNA extracted from gynoecia of *AGP18*-RNAi T2 lines and wild-type plants was blotted and hybridized with a portion of the *AGP18* cDNA. The blot was rehybridized with a probe specific to the constitutively expressed microRNA 39 (*miR39*) as a control. nt, nucleotides.

(D) Localization of *AGP18* mRNA in developing ovules at the functional megaspore stage. In situ hybridization with specific *AGP18* digoxigenin-labeled antisense probes was performed on gynoecia of both silenced (*AGP18*-RNAi T<sub>2</sub>-12; bar = 8.5  $\mu$ m) and wild-type plants (bar = 10  $\mu$ m). FM, functional megaspore.



sufficient quantities of total RNA were available, including lines having strong fertility defects as T2-12 and T2-58 but not in wild-type plants. Finally, to determine if a decrease in transcript levels was associated with a decrease in *AGP18* expression within the ovule or the anther, we performed in situ hybridization in selected lines showing the lowest levels of *AGP18* transcript. As shown in Figure 4D, no *AGP18* mRNA could be detected in the young ovule at the functional megaspore stage of *AGP18*-RNAi transformants, indicating that the normal expression of *AGP18* during female gametogenesis is severely impaired in *AGP18*-RNAi lines.

#### Posttranscriptional Gene Silencing Is Specific to *AGP18*

Plant transformation with RNAi vectors targeting a conserved gene family has been shown to often result in simultaneous posttranscriptional gene silencing of several family members. In Arabidopsis, 16 genes are predicted to encode the protein backbones of classical AGPs (Schultz et al., 2000). *AGP17* and *AGP19* encode classical AGPs with 56 and 42% amino acid similarity to *AGP18*, respectively. All three proteins are the only Arabidopsis AGP members containing a Lys-rich domain. At the DNA level, *AGP17* and *AGP19* share 55 and 52% homology with *AGP18* in the 740-bp cDNA fragment that was used to generate the RNAi construct. To determine if the transcript levels of any of these two genes were also decreased in *AGP18*-RNAi lines, total RNA extracted from developing gynoecia of three T2 *AGP18*-RNAi lines showing decreased levels of *AGP18* mRNA accumulation were used to perform RT-PCR analysis. As shown in Figure 5, both *AGP17* and *AGP19* are expressed in the gynoecia of wild-type plants; however, none of the T2 *AGP18*-RNAi lines analyzed showed a significant decrease in either *AGP17* or *AGP19* expression. In lines T2-12 and T2-58, no amplification signal could be detected after blotting RT-PCR gels and hybridizing with the corresponding *AGP18* probe, confirming that in these lines *AGP18* expression is almost completely silenced. These

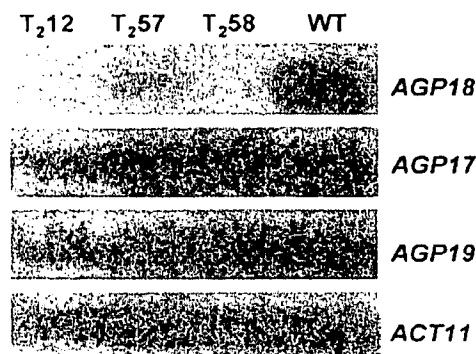


Figure 5. Posttranscriptional Gene Silencing Is Specific to *AGP18*.

RNA extracted from developing gynoecia of selected *AGP18*-RNAi T2 lines (T2-12, T2-57, and T2-58) was used for cDNA synthesis. PCR amplification was performed with primers specific to *AGP17*, *AGP18*, or *AGP19* (Schultz et al., 2002) using as a template samples corresponding to the same cDNA synthesis. Agarose gels were blotted on nitrocellulose membranes and probed with a corresponding AGP probe. Wild-type cDNA and amplification of *ACT11* were used as positive controls.

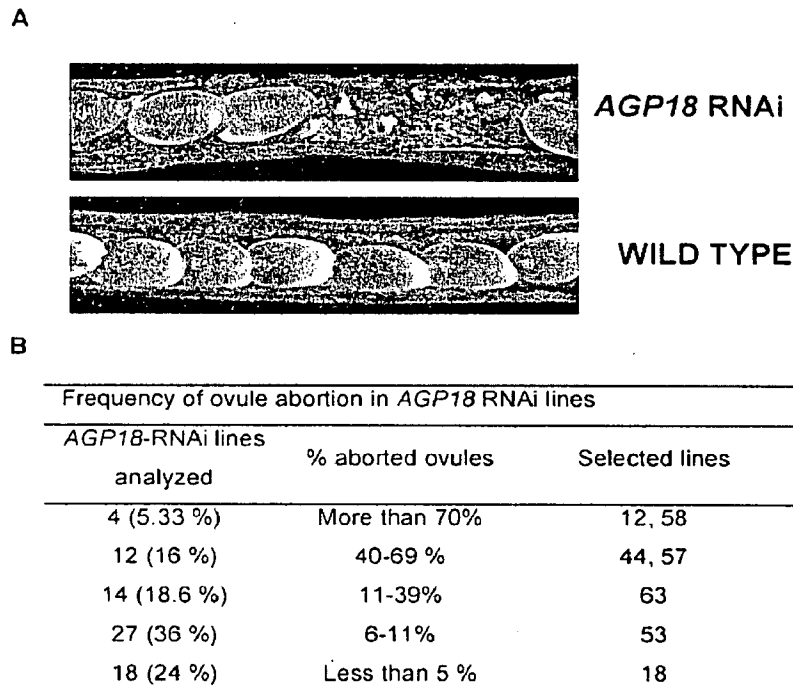
results demonstrate that in *AGP18*-RNAi lines posttranscriptional gene silencing is specific to *AGP18*.

#### Ovule Abortion in *AGP18*-RNAi Lines Is Controlled at the Sporophytic and Gametophytic Levels

As shown in Figure 6A, the siliques of *AGP18*-RNAi T2 lines contain a variable number of aborted ovules that do not show signs of early seed formation. This defect can also be observed in the form of empty spaces within siliques of self-fertilized flowers from *AGP18*-RNAi plants. Gametophytic defects affecting the female gametophyte but not the male are expected to show a decrease in seed set of ~50%. As shown in Figure 6B, T2 lines showed a wide range of frequencies of ovule abortion, with >20% showing a frequency significantly >50% and four lines showing >70% of aborted ovules. To determine the nature of the reproductive defect found in RNAi lines, we conducted reciprocal crosses between lines showing >50% ovule abortion and wild-type plants. When T2 lines were used as female parents, the same percentage of ovule abortion was obtained for all lines tested (data not shown); however, in crosses where T2 lines were used as male parents, full fertility was recovered, suggesting that the sterility defect is female specific.

DNA gel blot analysis was used to determine that the number of RNAi T-DNA insertions present in the genome of T2 lines varied between 1 and 6 (data not shown). Because the T-DNA RNAi construct used is marked with a *BASTA* herbicide resistance marker, its segregation pattern can easily be followed in seedlings. We characterized the segregation of *BASTA* resistance in a group of *AGP18*-RNAi lines showing high levels of ovule abortion but different numbers of T-DNA insertions. A summary of these results are presented in Table 1. Interestingly, a single T-DNA RNAi insertion was present in the genome of T2-12, a line showing 74.9% of aborted ovules and the highest levels of *AGP18* silencing in female reproductive organs. As shown in Table 1, T2-12 segregated *BASTA*-resistant (*BASTA*<sup>r</sup>) and *BASTA*-sensitive (*BASTA*<sup>s</sup>) seedlings in a distorted ratio of 1:1 *BASTA*<sup>r</sup>:*BASTA*<sup>s</sup> ( $\chi^2 = 2.41 < \chi^2_{0.05(1)} = 3.84$ ) as compared with 3:1 expected for normally transmitted insertions. In addition, no homozygous individuals have been identified in the T4 progeny resulting from self-pollination of 20 heterozygous T3-12 plants, suggesting that the transgene is poorly or not transmitted through the gametophytic phase of the Arabidopsis life cycle. The frequency of aborted ovules and the distorted segregation ratio in T2-12 suggests that posttranscriptional silencing of *AGP18* is affecting female reproductive development both at the sporophytic and gametophytic levels. In lines containing more than one T-DNA RNAi insertion, the number of *BASTA*<sup>r</sup> seedlings is significantly increased, suggesting that not all insertions are abnormally transmitted through the gametophytic phase and that the degree of semisterility found in *AGP18*-RNAi lines is not directly proportional to the number of introduced T-DNA insertions. These results are in agreement with recent estimations of the efficiency of RNAi in transgenic plants (Kerschen et al., 2004).

To determine if fertility defects were consistently inherited, we quantified the sterility phenotype during the first three consecutive generations of four selected lines. As shown in



**Figure 6.** Siliques of *AGP18*-RNAi Lines Show Aborted Ovules.

(A) Micrographs of *AGP18*-RNAi and wild-type siliques. The asterisks indicate the aborted ovules observed in *AGP18*-RNAi lines.

(B) An average of 250 ovules was scored for each *AGP18*-RNAi line. A  $\chi^2$  statistical analysis showed that lines having >5% ovule abortion were significantly different from the wild type (2.5% ovule abortion).

Table 2, the percentage of aborted ovules is maintained in the T3 generation; however, a small decrease in the frequency of ovule abortion was observed in all lines evaluated. Statistical analysis of variance showed that this decrease is not statistically significant during the generations tested.

#### Female Gametophyte Development Is Defective in *AGP18*-RNAi Lines

To determine the cellular nature of the defect, whole-mounted cleared anthers and ovules were analyzed during male and female reproductive development. We initially determined the terminal phenotypes of all *AGP18*-RNAi lines by examining mature organs at developmental stages in which male and female gametogenesis have normally resumed and fully differentiated cellularized gametophytes are already formed. All 58 lines examined had a variable proportion of ovules showing a similar abnormal phenotype but no defects in pollen formation. Our observations are summarized in Figure 7. In the large majority of defective ovules, a single conspicuous cell with a centrally located nucleus was present in the nucellus. In all lines, the conspicuous cell did not show signs of degeneration, and the adjacent nucellar tissue was not reabsorbed; in rare cases, its nucleus was not observed. To determine the developmental stage at which female gametophyte development

first departs from the wild type, we analyzed female gametogenesis at earlier developmental stages in ovules of line T2-12 (showing 74.9% of ovule abortion). All ovules of T2-12 undergo normal and synchronized megasporogenesis. Shortly after the differentiation of the functional megaspore (Figures 7A and 7E), a minority of ovules divide mitotically and give rise to two nuclei located at opposite ends of an enlarged cell showing a central vacuole. This type of ovule does not show differences with wild-type development (Figure 7B). By contrast, within the same gynoeceum, the nucellus of the majority of ovules contains a single cell that closely resembles the differentiated functional megaspore but that shows no signs of subsequent enlargement or vacuolization (Figure 7F). At later stages of development, whereas some ovules undergo two additional divisions and form a normal female gametophyte identical to the wild type (Figure 7C), the majority contains a single cell that does not divide mitotically (Figures 7G and 7H). To determine if the frequency of aborted ovules scored in dissected siliques is similar to the frequency of ovules showing abnormal female gametophyte development, we quantified the number of ovules showing a single conspicuous cell in the nucellus of fully differentiated ovules. The results are shown in Table 3. For all lines examined, there is a close correlation between the two values, suggesting that defects in female gametophyte development are sufficient to explain the abnormal phenotype observed in *AGP18*-RNAi lines.

**Table 1.** Number of T-DNA Insertions, Segregation Analysis, and Percentage of Ovule Abortion in Selected T2 *AGP18*-RNAi Lines

Line	Number of Insertions	BASTA <sup>1</sup>	BASTA <sup>2</sup>	Ratio	Viable Ovules	Aborted Ovules	Percentage of Ovule Abortion
T2-12	1	127	153	1.0:1.2	62	185	74.90
T2-57	2	143	53	2.6:1.0	100	125	55.55
T2-58	2	186	15	12.4:1.0	67	167	71.36
T2-44	5	182	46	3.9:1.0	125	99	49.01

### The Functional Megaspore Does Not Initiate Female Gametogenesis in *AGP18*-RNAi Ovules

To determine the identity of the cell that persists in the nucellus of defective ovules, we took advantage of additional enhancer detection and gene trap lines that show GUS expression in specific cells of the developing female gametophyte. Some of them represent ideal molecular markers to conduct crosses with *AGP18*-RNAi lines showing a high proportion of defective ovules. Figure 8 illustrates the pattern of GUS expression obtained in ovules of F1 plants resulting from crosses of line *AGP18*-RNAi T2-12 with individuals homozygous either for the ET499 or for the ET2209 enhancer detection element. In ET499, GUS is only expressed in the functional megaspore and not in the three dying megaspores or at earlier stages of megasporogenesis (Figures 8A to 8C; J.-P. Vielle-Calzada and U. Grossniklaus, unpublished results). This observation was confirmed in 100 whole-mounted and cleared ovules of homozygous ET499 plants. Other enhancer detection lines show GUS expression in the dying megaspores but not in the functional megaspore (Figure 8D), indicating that ET499 is an appropriate marker to characterize megaspores that have acquired a functional identity at the end of megasporogenesis. By contrast, ET2209 shows GUS expression at the onset of the second haploid mitotic division of the uncultured female gametophyte and subsequently in all haploid differentiated cells: the synergids, the egg cell, the central cell, and the antipodals (Figure 8G; Vielle-Calzada et al., 2000). In defective *AGP18*-RNAi/ET499/ F1 ovules, GUS expression is restricted to the conspicuous cell that persists in the nucellus after meiosis (Figures 8E and 8F), suggesting that in *AGP18*-RNAi lines female gametophyte development is arrested after the differentiation of the functional megaspore. By contrast, defective *AGP18*-RNAi/ET2209/ F1 do not show GUS expression (Figure 8H), indicating that the arrested functional megaspore does not acquire the identity of a multinucleated female gametophyte or of any of the haploid gametophytic cells. These results indicate that defective *AGP18*-RNAi ovules fail to undergo haploid mitosis after differentiation of the functional megaspore.

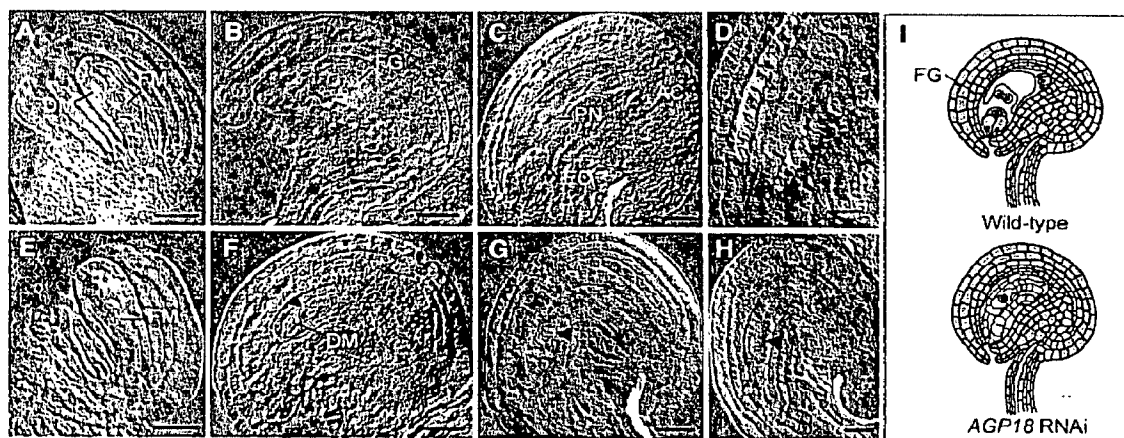
**Table 2.** Inheritance of Ovule Abortion in *AGP18*-RNAi Lines

Generation	Line			
	12	44	57	58
T1	74.9% (247)	44.1% (224)	55.50% (225)	71.36% (234)
T2	73.2% (220)	45.2% (271)	54.90% (232)	74.00% (255)
T3	67.5% (234)	39.0% (230)	46.15% (244)	70.80% (222)

### DISCUSSION

Here, we report the successful use of RNAi-induced posttranscriptional silencing to inactivate the *AGP18* gene and show that it plays an essential role during the initiation of female gametogenesis in Arabidopsis. *AGP18* encodes a classical AGP shown to be expressed in cells that spatially and temporally defines the sporophytic to gametophytic transition, but also during early stages of embryogenesis. More than 77% of independent transgenic Arabidopsis lines expressing the *AGP18*-RNAi construct showed moderate to severe fertility defects reminiscent of semi-sterile gametophytic mutants in Arabidopsis. Although in other experiments RNAi-dependent silencing is not always associated with mRNA turnover (Kerschen et al., 2004), all lines tested showed a decrease in *AGP18* transcript accumulation during female reproductive development in the T2 generation. In at least three lines, *AGP18* expression was almost completely suppressed. T2 lines with fertility defects showed ovules impaired in female gametogenesis but normal male gametophytic development and pollen formation. The use of molecular markers expressed at key stages of female gametogenesis determined that in defective ovules meiosis gives rise to a differentiated functional megaspore that is unable to give rise to a two-nucleate female gametophyte.

In plants developing a female gametophyte of the Polygonum type, megasporogenesis ends with the initiation of the haploid phase of the life cycle during the mitotic division of the functional megaspore nucleus (Huang and Russell, 1992). Although little is known about cellular communication during early ovule development, the interaction between sporophytic and gametophytic tissues has been suggested to be essential for female gametogenesis. For example, the isolation of the meiotic precursors and young tetrads by the accumulation of callosic walls has been interpreted as an interfacial reaction leading to the necessary separation of the two generations and the consequent protection of the haploid phase in ferns, mosses, and flowering plants (Dickinson, 1994; Bell, 1995). In Arabidopsis, the deposition of callose in dying meiotic products separates these cells from the functional megaspore (Webb and Gunning, 1990); however, the frequent formation of plasmodesmata connecting the functional megaspore to its adjacent nucellar cells indicates that cell-to-cell communication at the sporophytic-gametophytic transition is important during female gametophyte development (Bajon et al., 1999). In wild-type plants of Arabidopsis, several changes occur during the cytoplasmic maturation of the functional megaspore, including the polarized enlargement of the cell after the micropylar chalazal axis, the formation of a central vacuole, and the concomitant division of the nucleus (Webb and Gunning, 1990;



**Figure 7.** Female Gametophyte Development Is Defective in Ovules of *AGP18*-RNAi Lines.

Wild-type and *AGP18*-RNAi T2 gynoeceia were fixed, cleared, whole mounted, and viewed under Nomarsky optics.

(A) to (D) Development of wild-type ovules.

(A) Functional megaspore in a developing ovule.

(B) Female gametophyte at the two-nucleate stage.

(C) Mature female gametophyte.

(D) Young embryo at the two-cellular stage.

(E) to (H) Development of *AGP18*-RNAi T2-12 defective ovules.

(E) Functional megaspore in the developing T2-12 ovule.

(F) Arrested cell in defective T2-12 mature ovule (arrowhead); normal ovules in the same gynoeceium are at the two-nucleate stage.

(G) Arrested cell in defective T2-12 mature ovule (arrowhead); normal ovules in the same gynoeceium contain a mature female gametophyte.

(H) Arrested cell in T2-12 mature ovule; normal ovules in the same gynoeceium contain seeds undergoing early stages of embryogenesis.

(I) Schematic representation compares a mature wild-type ovule to a mature *AGP18*-RNAi T2-12 defective ovule.

FM, functional megaspore; DM, degenerating megaspore cells; EC, egg cell; AC, antipodal cells; FG, female gametophyte; PN, polar nuclei. Arrowheads indicate the presence of an arrested cell at the one-nucleate stage. Bars = 20  $\mu$ m.

Grossniklaus and Schneitz, 1998; Schneitz, 1999). No signs of cell enlargement or initial vacuolization were detected in arrested functional megaspores of *AGP18*-RNAi plants. In combination with results from expression analysis of molecular markers acting at specific stages of development, these observations indicate that the function of *AGP18* is required after differentiation of the functional megaspore for the initiation of female gametogenesis.

Several interpretations of the functional activity of *AGP18* during ovule development can be proposed based on the analysis of heterozygous *AGP18*-RNAi lines containing a single T-DNA insertion. Although T2-12 shows a 1:1 segregation ratio of *BASTA*<sup>r</sup> to *BASTA*<sup>s</sup> seedlings expected for defective traits causing gametophytic lethality, it also shows >70% of arrested female gametophytes, indicating that the gametophytic activity of RNAi-mediated silencing of *AGP18* is not sufficient to explain the defective phenotype. As shown by in situ hybridization, *AGP18* is expressed in the MMC and the adjacent nucellar cells; therefore, it is possible that, to ensure the initiation of female gametogenesis, the activity of *AGP18* is required in both sporophytic as well as gametophytic cells. Differences on the degree of penetrance of the RNAi effect at the diploid and haploid levels could explain the variable but incomplete sterility shown by all *AGP18*-RNAi lines. Recent studies suggest that the maximal reduction of target transcript levels is obtained in RNAi lines

containing a single T-DNA insertion (Kerschen et al., 2004). Although each target sequence is characterized by an inherent degree of susceptibility to RNAi-dependent silencing, a systematic study to assess the efficiency of posttranscriptional gene silencing in the gametophytic phase has not been conducted. Therefore, it is currently not possible to assess the effectiveness of *AGP18* silencing in the female gametophyte. A second possibility is that key RNA factors generated by RNAi-mediated *AGP18* silencing are produced at the diploid level and meiotically transmitted to a variable number of functional megaspores not carrying a T-DNA RNAi insertion; alternatively, these factors could be transported from adjacent nucellar cells to the functional megaspore via plasmodesmata. Although the transport of mRNA or proteins has not been reported in female meiotic products, detailed ultrastructural studies of megasporogenesis in *Arabidopsis* have shown that multiple plasmodesmata form between the functional megaspore and its adjacent nucellar cells (Bajon et al., 1999). Under this hypothesis, the function of *AGP18* could be strictly gametophytic; however, the non-fully penetrant effect of RNAi-mediated silencing factors generated at the diploid level would be responsible for the abortion of female gametophytes at a frequency significantly higher than 50%. A third alternative includes the possibility that gametophytic lethality in T2-12 results from differences in the degree of RNAi silencing mediated by the CaMV35S promoter. A potential lack

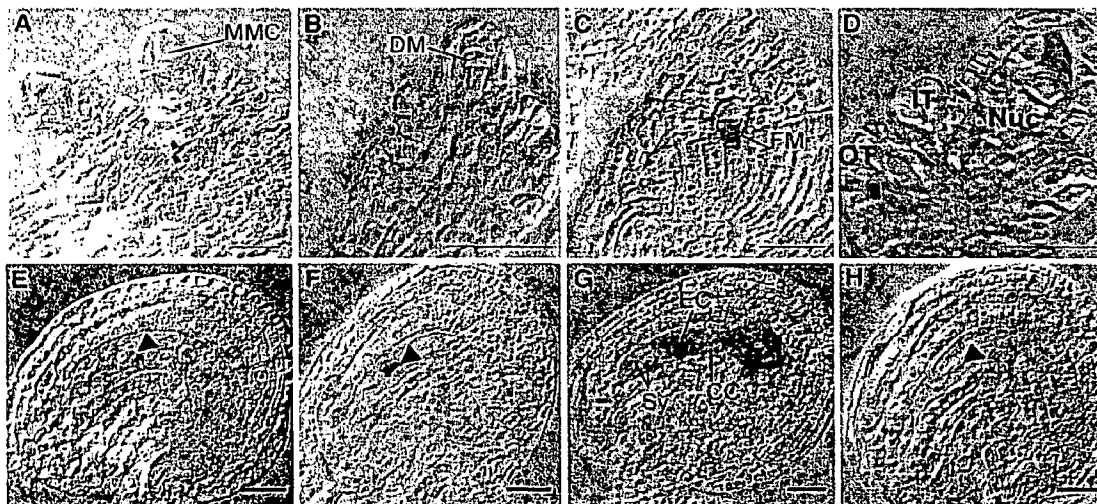
**Table 3.** Frequency of Ovules Showing an Arrested Cell Phenotype

<i>AGP18</i> -RNAi Line	Viable Ovules	Aborted Ovules	Ovules with Arrested Cells
T2-12	138 (27.7%)	185 (74.9%)	359 (72.23%)
T2-57	196 (43.3%)	125 (55.5%)	257 (56.73%)
Wild type	443 (99.4%)	6 (2.4%)	3 (0.60%)

of CaMV35S activity during microsporogenesis could partially explain the absence of pollen abnormalities associated with the expression of *AGP18* during anther development. To date, a detailed pattern of the CaMV35S promoter activity during male and female gametogenesis has yet to be reported in Arabidopsis. Although it is generally believed that this promoter is not active during the gametophytic phase (Bechtold et al., 2000), it is not clear at which developmental stage of the sporophytic to gametophytic transition its activity is no longer detected in either male or female gametes. Although this lack of quantitative information on the pattern of CaMV35S complicates the elucidation of the role played by *AGP18* during the alternation of

diploid and haploid phases, experiments showing that RNAi factors transmitted during meiosis can trigger posttranscriptional silencing in the female gametophyte support the hypothesis that the CaMV35S promoter can be used to successfully target gametophytically expressed genes (our unpublished results). The use of specific promoters to drive RNAi-mediated silencing exclusively in the gametophyte or in the nucellus should lead to the elucidation of the specific role of gametophytic and sporophytic *AGP18* activity during the initiation of female gametogenesis.

Classical AGPs play a role in mechanisms as distinct as cell division, cell expansion, or cell determination (Nothnagel, 1997; Schultz et al., 1998; van Hengel and Roberts, 2003). Monoclonal antibodies (MAbs) raised against AGP epitopes have been extensively used to investigate the cellular localization of AGPs (VandenBosch et al., 1989; Knox, 1992); however, these probes do not easily allow the elucidation of single AGP distribution patterns because they can recognize many different AGPs containing a conserved sugar epitope but different protein backbones. Elegant immunolocalization studies have shown that the establishment of a reproductive lineage in certain

**Figure 8.** The Functional Megaspore Does Not Initiate Female Gametogenesis in *AGP18*-RNAi Ovules.

Ovules of F1 plants resulting from crosses of line *AGP18*-RNAi T2-12 with individuals homozygous either for the ET499 or for ET2209 were either fixed and whole-mount cleared or processed for histochemical localization of GUS activity. Shown are patterns of GUS expression in ET499 (**A**) to (**C**), ET4127 (**D**), F1 plants resulting from the cross of *AGP18*-RNAi T2-12 and homozygous ET499 plants (**E**) and (**F**), ET2209 (**G**), and F1 plants resulting from the cross of *AGP18*-RNAi T2-12 and homozygous ET2209 plants (**H**).

(**A**) Ovules of ET499 at MMC not showing GUS expression.

(**B**) Ovules of ET499 showing absence of expression in all three dying megaspores.

(**C**) Functional megaspore showing GUS expression in ET499.

(**D**) Ovule of ET4127 showing GUS expression in a dying megaspore.

(**E**) Whole-mounted cleared ovule showing the phenotype observed in defective *ET499;AGP18*-RNAi T2-12 F1 ovules.

(**F**) GUS expression in the arrested functional megaspore of defective *ET499;AGP18*-RNAi T2-12 F1 ovules. The arrowhead shows the position of the arrested functional megaspore.

(**G**) Pattern of GUS expression in the mature female gametophyte of ET2209.

(**H**) Absence of GUS expression in defective *ET2209;AGP18*-RNAi T2-12 F1 ovules.

FM, functional megaspore; IT, inner integument; OT, outer integument; Nuc, nucellus; DM, dying megaspore; EC, egg cell; Sy, synergid cells; CC, central cell. The arrowheads indicate the arrested cells. Bars = 20  $\mu$ m.

species was associated with changes in the distribution of AGP epitopes (Knox et al., 1989; Pennell et al., 1992). In *Pisum sativum*, the determination of reproductive cells in male and female gametes is associated with the loss of a cell surface arabinose-containing epitope recognized by the Mab MAC207 (Pennell and Roberts, 1990). Interestingly, a second AGP epitope recognized by Mab JIM8 became detectable only during differentiation of anthers and ovules in *Brassica napus* (Pennell et al., 1991). During ovule development, this epitope was initially detected in the wall of the two- to four-nucleate female gametophyte, in nucellar cells adjacent to the developing female gametophyte, and later in the plasma membrane of all cells forming the egg apparatus, but not the central cell. The presence of this epitope was also detected in the tapetum, in developing pollen grains, and in the stem vasculature (Pennell et al., 1991). Our results show that *AGP18* is expressed in reproductive tissues, particularly in cell types that are involved in establishing the sporophytic to gametophytic transitions. During female gametophyte development, *AGP18* is initially expressed in the MMC, in all four meiotically derived megaspores, the functional megaspore, and the adjacent nucellar cells. *AGP18* expression persists in all female gametophyte cells except the central cell. During male gametophytic development, *AGP18* is expressed in pollen grains and the cells of the tapetum. Developing seeds also express *AGP18* during the first stages of embryogenesis; interestingly, abundant *AGP18* mRNA is detected at early stages of endosperm development, indicating that transcription in the central cell occurs only after double fertilization. We only detected additional *AGP18* expression in restricted clusters of companion cells present in the vasculature of the stem. The pattern of *AGP18* mRNA localization is almost identical to the pattern of localization of the epitope recognized by JIM8, strongly suggesting that an AGP encoded by a gene homologous to *AGP18* is detected by JIM8 in *B. napus*. The generation of *AGP18*-RNAi lines opens new possibilities for immunolocalization analysis with JIM8 and new antibodies raised against *AGP18* to further elucidate the function and distribution of AGPs during reproductive development.

Our results indicate that *AGP18* plays a crucial role during interactions between sporophytic and gametophytic cells in the young ovule and that these interactions are essential for the establishment of the female gametophytic phase in *Arabidopsis*. Although the nature of this communication has yet to be characterized at the genetic and molecular levels, the elucidation of the developmental function of a gene encoding a classical AGP creates new perspectives for the understanding of cell surface signaling and the molecular mechanisms that regulate sexual reproduction in flowering plants.

## METHODS

### Plant Material and Growth Conditions

The transposant line MET333 (*Arabidopsis thaliana* Heynh. var *Landsberg erecta*) was identified in a collection of enhancer detector lines generated in our laboratory, using the system implemented by Sundaresan et al. (1995), looking for expression patterns during female gametophyte de-

velopment. To select plants carrying the *Ds* transposon, 50 mg/L of kanamycin and 0.66 µg/mL of 1-naphthalenacetamide were added to MS solid medium (Sigma, St. Louis, MO). Resistant seedlings were transferred to soil and grown in a greenhouse under long-day conditions.

### Generation of RNAi Lines

A 740-bp fragment containing the first exon and a region of 5' untranslated region of *AGP18* was amplified by RT-PCR using RNA extracted from wild-type developing gynoecia using the following primers containing restriction sites as indicated in boldface: 5'-AATCTA-GAGGCGCGCCACGGCTACATCTGTCTGT-3' (*Xba*I and *Asc*I; sense primer) and 5'-AAGGATCCATTAAATATGTACCTGATCGTCGG-3' (*Bam*HI and *Swa*I; antisense primer). The PCR fragment generated with the sense/antisense primer combination was cloned in pCRII TOPO (Invitrogen, Carlsbad, CA) and subsequently digested with *Swa*I and *Asc*I restriction enzymes. The resulting DNA fragment was cloned using appropriate restriction sites in the silencing vector pFGC5941 (kindly donated by Carolyn Napoli and Rich Jorgensen, www.chromdb.org). The PCR fragment cloned in pCRII TOPO was digested with *Xba*I and *Bam*HI (to obtain the antisense fragment) and cloned in appropriate restriction sites of pFGC5941. The resulting pFGC5941 vector contained *AGP18* in both sense and antisense orientations separated by a chalcone synthase intron and under the control of the CaMV35S promoter. Four-week-old *Arabidopsis* plants (Columbia-0) were transformed by floral dipping as previously described (Clough and Bent, 1998). Seeds from *Agrobacterium tumefaciens*-treated plants were selected and directly grown under greenhouse long-day conditions (16 h light). Resistant seedlings were selected by spraying the herbicide BASTA (50 mg/L; Finale; AgrRvo, Montvale, NJ) three times each week for 2 weeks. Presence of *AGP18*-RNAi insertions was confirmed by PCR amplification on DNA extracted from seedlings. Seeds from mature plants were collected and plated onto MS medium supplemented with glufosinate ammonium (10 µg/mL; Crescent Chemical, Augsburg, Germany).

### RNA Analysis

For RNA gel blots, total RNA was extracted from developing gynoecia from selected *AGP18*-RNAi transformants and wild-type plants using Trizol (Invitrogen) and following the manufacturer's instructions. Fifty micrograms of RNA were separated in a 1.3% agarose gel containing 17% formaldehyde and blotted onto hybrid N<sup>+</sup> membranes. Blots were hybridized with random-primed (Amersham Biosciences, Buckinghamshire, UK) <sup>32</sup>P-labeled 810-bp probe corresponding to the complete *AGP18* cDNA (At4g37450) and an *ACT11* (At3g12110) probe generated by PCR using the following primers: ACT11-S (5'-TTCAACACTCCTGCCATG-3') and ACT11-AS (5'-TGCAAGGTCCAAACGCAG-3'). The temperature of hybridization was 65°C in Church's buffer.

For small RNA analysis, total RNA from developing gynoecia was enriched for low molecular weight RNAs using Hamilton's homogenization solution (Hamilton and Baulcombe, 1999) as described in Mette et al. (2000). Low molecular weight RNA was normalized by spectrophotometry to 100 µg/lane, separated by electrophoresis through 15% polyacrylamide, 7 M urea, 0.5× Tris-borate EDTA gel, and transferred to Zeta-Probe GT membranes (Bio-Rad, Hercules, CA). After transfer, membranes were cross-linked with 200 mJ of UV and baked at 80°C for 1 h. To detect small RNAs in *AGP18*-RNAi lines, an *AGP18* cDNA probe as described above was randomly labeled and hybridized at 62°C in Church's buffer. An oligonucleotide probe corresponding to the sequence of miR39 (5'-GATATTGGCGCGGCTCAAGCA-3'; Llave et al., 2002) was 5'-end labeled with [<sup>32</sup>P]ATP and hybridized as a loading control. For RT-PCR analysis, total RNA was isolated from developing gynoecia of wild-type and *AGP18*-RNAi lines using Trizol following the manufacturer's instructions. First-strand cDNA was synthesized using

2 µg of total RNA and Superscript II reverse transcriptase (Invitrogen). For semiquantitative RT-PCR, 3 µL of the first-strand cDNA reaction served as a template in a PCR reaction that used specific primers for *AGP18* (At4g37450), *AGP17* (At2g23130), *AGP19* (At1g68725), and *ACT11*. After estimating the amount of amplified DNA produced at different rounds of PCR cycles, we determined that 20 cycles ensured that the amplified product was proportional to the initial concentration of template present in the reaction. After electrophoresis on a 1% agarose gel and blotting into hybrid N<sup>+</sup> membranes, hybridization was performed with <sup>32</sup>P cDNA probes specific to each *AGP* gene tested and labeled with the random primer method (Amersham Biosciences). Hybridization was performed at 62°C as described (Maniatis et al., 1989). *AGP17* primers were as follows: sense (5'-GCTTTTAAGCCCGCCTGCTCC-3') and antisense (5'-CTG-AATACAAATGTGAGCTG-3'). *AGP19* primers were as follows: sense (5'-AAGTTGCACCAGTAATCAGCC-3') and antisense (5'-TCCTTTAAG-CTGATTTAAGGC-3'). *AGP18* primers were as follows: sense (5'-CACGCTTGGTTAACTCC-3') and antisense (5'-TTTTTCATCACT-GACAG-3').

#### Whole-Mount Preparations and Histological Analysis

Wild-type and *AGP18*-RNAi siliques were dissected longitudinally with hypodermic needles (1-mL insulin syringes) and fixed with FAA buffer (50% ethanol, 5% acetic acid, and 10% formaldehyde), dehydrated in increasing ethanol concentration, cleared in Herr's solution (phenol: chloral hydrate:85% lactic acid:xylene:oil of clove [1:1:1:0.5:1]), and observed on a Leica microscope (Wetzlar, Germany) under Nomarski optics. GUS staining assays for stages before fertilization were conducted as described by Vielle-Calzada et al. (2000). For developmental stages after fertilization, we used the protocol described by Köhler et al. (2003) with slight modifications. Longitudinally dissected siliques were fixed for 2 h at -20°C in 90% acetone and subsequently immersed in GUS staining buffer (10 mM EDTA, 0.1% Triton, 0.5 mM Fe<sup>2+</sup>CN, 0.5 mM Fe<sup>3+</sup>CN, 100 µg mL<sup>-1</sup> chloranphenicol, and 1 mg mL<sup>-1</sup> 5-bromo-4-chloro-3-indolyl-β-D-galactoside in 50 mM sodium phosphate buffer, pH 7.0) for 1 to 3 d at 37°C. The tissue was cleared in Hoyer's solution and observed under Nomarski optics.

#### In Situ Hybridization

Developing flower buds, developing flowers, isolated gynoecia, and siliques of wild-type and *AGP18*-RNAi T2-T12 plants were fixed in 4% paraformaldehyde and embedded in Paraplast (Fisher Scientific, Fair Lawn, NJ). Sections of 12-µm thickness were cut using a Leica microtome and mounted on ProbeOnPlus slides (Fischer Biotech, Pittsburgh, PA). A fragment of 180 bp that included a portion of the first exon of *AGP18* was amplified using sense (5'-CGACGATCAGGTACATTAG-3') and the antisense (5'-CATCACTGACAGATATGAA-3') primers and subsequently cloned in the pCRII TOPO vector (Invitrogen). The resulting construct was digested with *NotI* and *BamHI* to synthesize sense and antisense digoxigenin-labeled probes, respectively, and hybridization was conducted as described by Vielle-Calzada et al. (1999).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number NM119909 (At4g37450).

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## TECHNICAL ADVANCE

# A chemical-regulated inducible RNAi system in plants

Hui-Shan Guo<sup>1</sup>, Ji-Feng Fei<sup>1</sup>, Qi Xie<sup>1</sup> and Nam-Hai Chua<sup>2,\*</sup><sup>1</sup>Laboratory of Molecular Cell Biology, Temasek Life Sciences Laboratory, National University of Singapore, 1 Research Link, 117604 Singapore, and<sup>2</sup>Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

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\*For correspondence (fax +1 212 327 8327; e-mail chua@mail.rockefeller.edu).

## Summary

Constitutive expression of an intron-containing self-complementary 'hairpin' RNA (ihpRNA) has recently been shown to efficiently silence target genes in transgenic plants. However, this technique cannot be applied to genes whose silencing may block plant regeneration or result in embryo lethality. To obviate these potential problems, we have used a chemical-inducible Cre/loxP (CLX) recombination system to trigger the expression of an intron-containing inverted-repeat RNA (RNAi) in plants. A detailed characterization of the inducible RNAi system in transgenic *Arabidopsis thaliana* and *Nicotiana benthamiana* plants demonstrated that this system is stringently controlled. Moreover, it can be used to induce silencing of both transgenes and endogenous genes at different developmental stages and at high efficiency and without any detectable secondary effects. In addition to inducing complete silencing, the RNAi can be produced at various times after germination to initiate and obtain different degrees of gene silencing. Upon induction, transgenic plants with genetic chimera were obtained as demonstrated by PCR analysis. Such chimeric plants may provide a useful system to study signaling mechanisms of gene silencing in *Arabidopsis* as well as other cases of long-distance signaling without grafting. The merits of using the inducible CLX system for RNAi expression are discussed.

**Keywords:** inducible, constitutive, RNAi, dsRNA, DNA excision, Cre/loxP.

## Introduction

RNA-mediated gene silencing is a conserved mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger sequence-specific degradation of homologous mRNA. dsRNA has been used as a powerful tool for the investigation of RNA silencing in a variety of organisms, such as RNA interference (RNAi) in *Caenorhabditis elegans* (Fire *et al.*, 1998) and mammalian cells (Paddison *et al.*, 2002; Sui *et al.*, 2002) and post-transcriptional gene silencing (PTGS) in plants (Chuang and Meyerowitz, 2000; Waterhouse *et al.*, 1998). In plants and *C. elegans*, RNA silencing involves two steps: (i) a local induced silencing, including an initial processing of the triggering dsRNA into short interfering RNA (siRNA) of 21–25 nt (Elbashir *et al.*, 2001; Hamilton and Baulcombe, 1999) and (ii) a systemic spread of the silencing signal throughout the entire organism (Voinnet *et al.*, 2000; Winston *et al.*, 2002). The presence of a spliceable intron in the transgene encoding the dsRNA appears to enhance the silencing efficiency (Smith

*et al.*, 2000; Wesley *et al.*, 2001). Constitutive expression of intron-containing self-complementary 'hairpin' RNA (ihpRNA) constructs can induce PTGS with almost 100% efficiency when directed against viruses or endogenous genes (Smith *et al.*, 2000).

The completion of the sequencing of the *Arabidopsis* genome has uncovered a large number of genes with unknown functions. Potentially, the dsRNA-mediated gene silencing technique can be used to investigate the functions of these genes. The most effective silencing, brought about by intron-containing dsRNA, would produce phenotypes resembling those of the null alleles of the target genes. If the target gene is required for basic cell function or development, constitutive dsRNA-mediated silencing of the gene may produce detrimental effects or even cause plant lethality resulting in no recovery of transgenic plants for investigation. This problem can be circumvented somewhat by inducing gene silencing, using either *Agrobacterium*

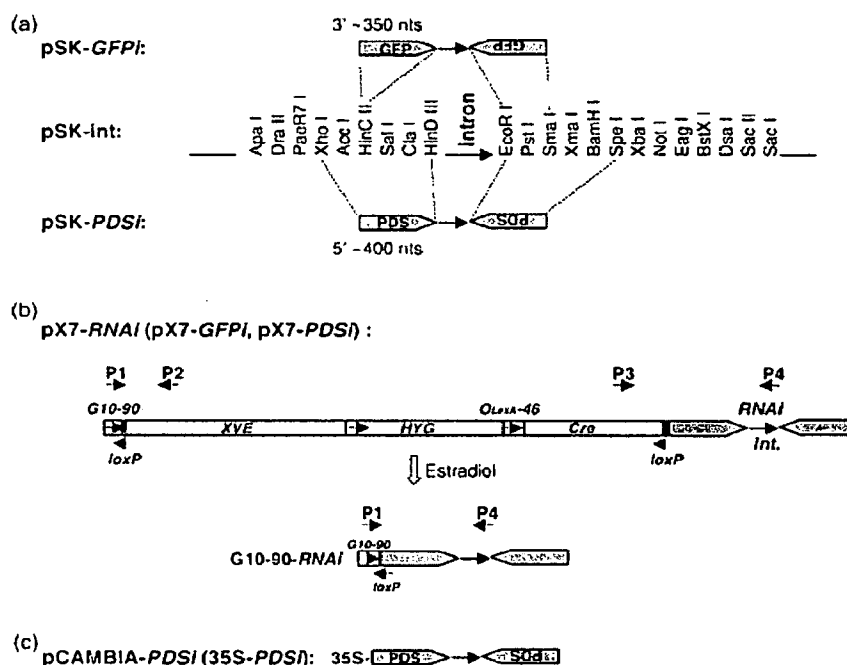


Figure 1. Schematic diagrams of the inducible and constitutive RNAi constructs.

(a) A schematic diagram of an intron-containing intermediate vector pSK-int with multiple restriction sites on both arms of the intron. DNA fragments encoding sense and antisense RNA (approximately 350 nt of 3' *GFP* or approximately 400 nt of 5' *PDS*, respectively) were cloned into pSK-int at 5' and 3' arms of the intron in appropriate restriction sites indicated by dotted lines, resulting in pSK-GFPi and pSK-PDSi.

(b) A schematic diagram showing structural features of the inducible pX7-RNAi construct and *Cre/loxP*-mediated DNA recombination (see Zuo *et al.*, 2001 for details). *XVE*, a chimeric transactivator containing the regulator domain of an estrogen receptor (Zuo *et al.*, 2000); *HYG*, a hygromycin-resistance marker; *Cre*, the bacteriophage P1 Cre recombinase with an intron (Zuo *et al.*, 2001); *loxP*, specific recognition sites of Cre; *OlexA-46*, eight copies of the *LexA* DNA binding site fused to the -46 CaMV 35S promoter; *G10-90*, a strong, synthetic, constitutive promoter; *int*, actin 11 intron (ATU27981, nt 1957–2111); *RNAi*, DNA sequences encoding the intron-containing inverted-repeat RNAs; *G10-90-RNAi*, the reconstituted transcription unit derived from *Cre/loxP*-mediated DNA recombination after inducer treatment. Arrows inside transcription units indicate the direction of transcription. P1–4 denote primers used for PCR analysis shown in Figure 5(c).

(c) A fragment of PDS-int-PDS was cloned between the 35S promoter and the terminator in the binary plasmid pCambia 1300 (AF234296) to give 35S-PDSi, a constitutive RNAi construct.

infiltration (Voinnet and Baulcombe, 1997) or virus-derived vectors (Dalmay *et al.*, 2000; Peele *et al.*, 2001; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002). However, these two methods have limited application in functional genomics because of their transient nature, and their silencing effects are not heritable.

In this paper, we describe the development of an inducible gene silencing system using intron-containing, inverted-repeat RNA (referred to as inducible RNAi). The chemical-inducible system we use here is the CLX system, an XVE-based (XVE for *LexA*-VP16-ER) (Zuo *et al.*, 2000), site-specific DNA recombination mediated by *Cre/loxP* (Zuo *et al.*, 2001). The *Cre* expression is placed under the control of the chimeric transcription factor XVE, whose activity is strictly regulated by estrogens. Upon induction by 17 $\beta$ -estradiol, *Cre/loxP*-mediated recombination leads to activation of the RNAi transcription cassette by bringing it immediately downstream of the constitutive *G10-90* promoter (Zuo *et al.*, 2001; Figure 1). Compared to other inducible systems, the most significant feature of the CLX system is that it is stringently controlled, and upon induction, it

produces DNA recombination with high efficiency (for a review, see Hare and Chua, 2002; Ow, 2001).

Here, we show that the inducible RNAi system can be used to silence, with high efficiency, the expression of a *GFP* transgene and an endogenous phytoene desaturase (*PDS*) gene in *Nicotiana benthamiana* as well as in *Arabidopsis thaliana*. Upon induction at seed germination or post-germination stages, the efficiency and effectiveness of *PDS* silencing are comparable to those obtained with a 35S-RNAi construct. A stable and reproducible inducible RNAi phenotype was obtained in subsequent transgenic generations. The merits of the inducible RNAi system for silencing of endogenous genes are discussed.

## Results

### Silencing of a *GFP* transgene

To evaluate the function of the inducible RNAi system, the construct pX7-GFPi (Figure 1b) was introduced into a

transgenic *N. benthamiana* line carrying a 35S-*GFP* transgene (GFP-16c) (Ruiz *et al.*, 1998) by *Agrobacterium*-mediated transformation. T<sub>1</sub> seeds from 12 transgenic lines (GFP-16c/pX7-*GFPi*, named as 16c-*GFPi*) were germinated on the inductive medium (MS + 2  $\mu$ M 17 $\beta$ -estradiol) containing hygromycin (hyg). After 2 weeks, eight lines showed uniform red fluorescence under UV light, which was caused by chlorophyll autofluorescence in the absence of GFP accumulation. Some red seedlings stopped growing upon continued incubation on the selective medium, presumably because of the complete Cre/*loxP*-mediated excision of the hygromycin-resistance gene upon induction. The remaining four lines initially displayed a mixture of red/green fluorescence after 2 weeks of treatment with the inducer, but propagation of the red color to the entire plant was observed in these lines 2 weeks after they were transferred from the inductive medium to the soil (Figure 2a, left). No difference was observed between 16c-*GFPi* seedlings germinated on the selective medium in the absence of the inducer (MS + hygromycin) and GFP-16c control plants, as judged by the degree of green fluorescence (Figure 2b, right).

Northern blots were analyzed with total RNA from treated red fluorescent 16c-*GFPi* seedlings, untreated green fluorescent 16c-*GFPi* seedlings and GFP-16c control seedlings. *GFP* mRNA was almost undetectable in red 16c-*GFPi* seedlings (Figure 2b, upper panel, +), as compared with GFP-16c control (0) or untreated 16c-*GFPi* seedlings (-). *GFP*-related siRNA, which is a key component of RNA silencing, was detected in red seedlings using a radiolabeled *GFP*-specific probe (Figure 2b, lower panel, +), whereas no *GFP*-related siRNA could be detected in GFP-16c control or untreated 16c-*GFPi* green seedlings (0 and -). Both fluorescence and RNA analyses indicated silencing of the *GFP* transgene in 16c-*GFPi* plants upon treatment with 17 $\beta$ -estradiol.

A transgenic line of *A. thaliana* (ecotype C24), which showed constitutive expression of a 35S-*GFP* transgene (Dalmay *et al.*, 2000) was transformed with the pX7-*GFPi* construct to test the inducible RNAi system. Twenty-nine T<sub>1</sub> independent lines (named as At-*GFPi*) were obtained by floral dip transformation. Upon germination on the inductive medium, all 29 At-*GFPi* lines displayed red fluorescence, indicating silencing of the *GFP* transgene. By contrast, all seedlings germinated on the medium without the inducer showed uniform green fluorescence, indicating *GFP* expression (data not shown). Northern blots were analyzed with total RNA (Figure 2c, upper panel) and *GFP*-related siRNA (Figure 2c, lower panel) from treated, red fluorescent At-*GFPi* seedlings (Figure 2c, +) and untreated, green fluorescent At-*GFPi* seedlings (Figure 2c, -). Similar results were obtained as with the *N. benthamiana* 16c-*GFPi* plants, providing molecular evidence for silencing of the *GFP* transgene in At-*GFPi* plants upon 17 $\beta$ -estradiol treatment.

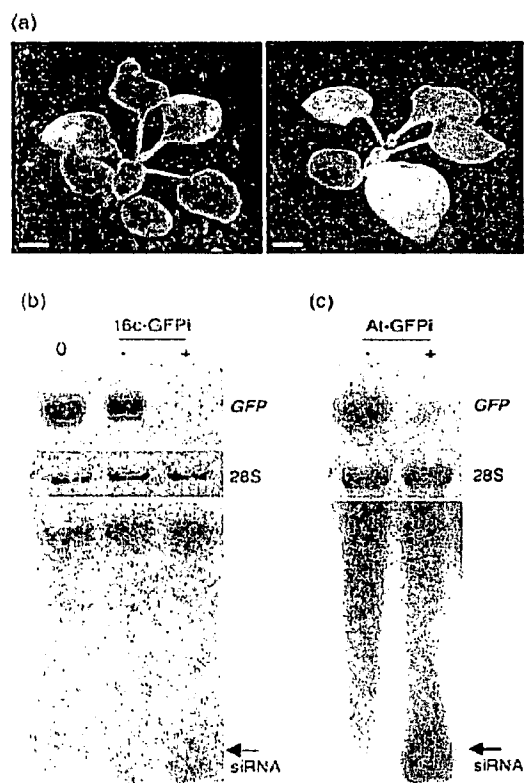
We used 15 At-*GFPi* lines for further investigation of 17 $\beta$ -estradiol-induced silencing at post-germination stage. Two-week-old T<sub>1</sub> seedlings germinated on the selective medium in the absence of the inducer were transferred to fresh MS medium. All seedlings at this stage continued to display green fluorescence. However, when seedlings were transferred to the inductive medium, 10 lines displayed strong inducible *GFP* silencing as indicated by their uniform red fluorescence after 1-week induction (data not shown). The remaining five lines showed varying initiation of *GFP* silencing after 1-week induction. Further incubation with the inducer up to 2 weeks resulted in complete *GFP* silencing as reflected by the uniform red fluorescence in all these plants.

#### *Efficient inducible silencing of an endogenous PDS gene in Arabidopsis thaliana*

We chose the phytoene desaturase (*PDS*) gene of *A. thaliana* and *N. benthamiana* to test the ability of the inducible RNAi system to silence endogenous genes. The *PDS* gene was selected because loss of the phytoene desaturase enzyme blocks carotenoid synthesis culminating in a photobleaching phenotype because of photo-oxidation of chlorophylls (Ruiz *et al.*, 1998). This visible phenotype facilitated visual monitoring of the induction process of *PDS* silencing. The constructs pX7-*PDSi*(At) and pX7-*PDSi*(Nb) (Figure 1b) were transformed into *A. thaliana* (ecotype Columbia) and *N. benthamiana*, respectively. In addition, pCambia-*PDSi*(At) (Figure 1c) containing a 35S-*PDSi*(At) was also transformed into *A. thaliana* (ecotype Columbia).

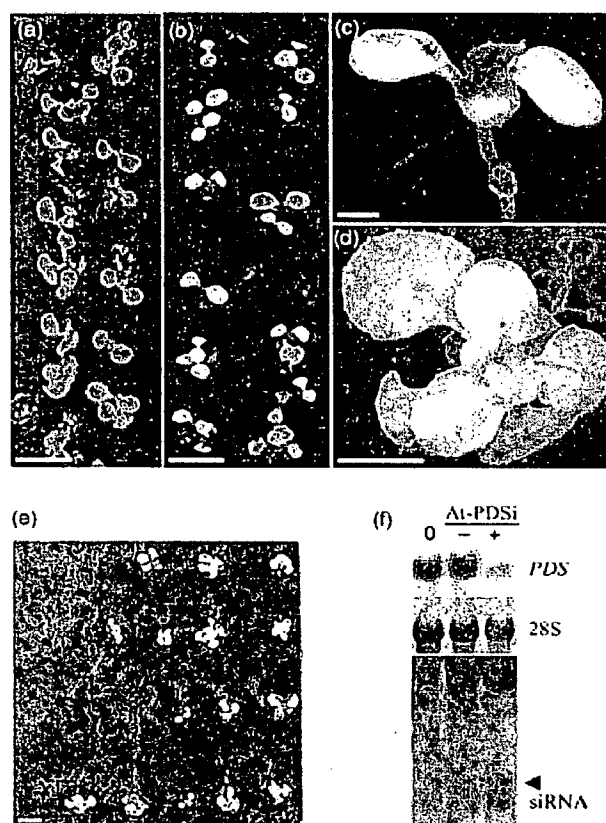
We tested 35 lines of putative transgenic *A. thaliana* carrying the 35S-*PDSi*(At) transgene by virtue of their ability to grow on the selective medium. Thirty-two lines (35S-*PDSi*(At)) displayed the photobleaching phenotype. Most lines appeared near-white and stopped growing (data not shown) after 4–6 weeks on the culture medium. Only two lines that displayed varying green patches in their bleached leaves survived. Seedlings of these two lines exhibited abnormal development and poor fertility, and produced only a small amount of seeds.

Eighty-one independent *A. thaliana* transgenic lines transformed with pX7-*PDSi*(At) were obtained. In the absence of the inducer, all transgenic T<sub>1</sub> lines (At-*PDSi*) displayed normal development and fertility. T<sub>2</sub> seeds from 12 independent T<sub>1</sub> lines were germinated on the selective medium in the absence or presence of the inducer. All T<sub>2</sub> seedlings grew with normal phenotype on the medium in the absence of the inducer (Figure 3a). However, in the presence of the inducer, seedlings of all the 12 T<sub>2</sub> At-*PDSi* lines showed uniform photobleaching phenotype in the cotyledons at 6–9 days post-induction (Figure 3b,c). Similar to the 35S-*PDSi*(At) lines, most of these seedlings



**Figure 2.** 17 $\beta$ -estradiol-inducible *GFP* silencing in *Nicotiana benthamiana* transgenic line 16c-GFPi and *Arabidopsis thaliana* line At-GFPi. (a) 16c-GFPi plants were first germinated on an inductive (left) or selective (right) medium for 2 weeks, before being transferred to soil, and photographed under UV light at 2 weeks after transfer. Left, an induced 16c-GFPi plant exhibited uniform red fluorescence in the whole plant. Note that in this plant initiation of *GFP* silencing was indicated by a mixture of red/green fluorescence. Right, an untreated 16c-GFPi plant showing green fluorescence. Scale bar = 0.5 cm. (b, c) Northern blot analysis of induced silencing in 16c-GFPi plants (b) and At-GFPi plants (c). Total high (top and middle panels) and low (lower panel) molecular weight RNAs were extracted from 2-week-old red fluorescent seedlings after 17 $\beta$ -estradiol induction (+), untreated green fluorescent seedlings (–), and GFP-16c control plants (0). Equal RNA loading (5  $\mu$ g per leaf) was monitored by methylene blue staining of the 28S RNA (middle panel), and the blot was probed with a *GFP*-specific sequence (top panels). Bottom panels show the blot for siRNA, which was hybridized with a  $^{32}$ P-labeled transcript corresponding to the 3'-terminal region (350 nt) of *GFP*. Each lane contained 50  $\mu$ g RNA. Arrow indicates the position of a 25-base DNA oligonucleotide.

stopped growing within 4–6 weeks (Figure 3d). Northern analysis showed that endogenous *PDS* mRNA levels were significantly reduced in bleached leaves of treated At-PDSi lines (Figure 3f, upper panel, +), but readily detected in untreated lines (Figure 3f, upper panel, –) and WT control seedlings (Figure 3f, upper panel, 0), indicating that the photobleaching phenotype resulted from silencing of the endogenous *PDS* gene. *PDS*-related siRNA was detected in treated, bleached At-PDSi seedlings (Figure 3f, lower



**Figure 3.** Inducible *PDS* silencing in transgenic *Arabidopsis thaliana* plants (At-PDSi) at seed germination stage. (a–d) At-PDSi seeds of line 2 were germinated on selective media in the absence (a) or presence of an inducer (b, c, d). (b) Seedlings with induced *PDS* silencing showed a uniform photobleaching phenotype in the cotyledons at 6 days. (c, d) *PDS* silencing was seen in the first pair of true leaves as well as the leaves that developed later (c, 9 days, d, 28 days). Scale bars = 0.5 cm in (a, b, d) and 0.1 cm in (c). (e) Seeds of At-PDSi line 1 (a single transgenic locus) were germinated on MS in the presence of 17 $\beta$ -estradiol without the selective antibiotic hygromycin. Fifteen out of 19 plants showed induced *PDS* silencing. The photograph was taken at 29 days post-induction. Scale bar = 0.5 cm. (f) Northern analysis of inducible *PDS* silencing in At-PDSi plants. Total high (top and middle panels) and low (lower panel) molecular weight RNAs were extracted from WT Columbia seedlings (0), uninduced At-PDSi control seedlings (–), and a mixture of induced At-PDSi seedlings, which showed a photobleaching phenotype (+). The blot (top panel) was probed with a *PDS*-specific sequence corresponding to the 3' region for detection of the endogenous *PDS* mRNA. Each lane contained 5  $\mu$ g RNA, and 28S RNA visualized by methylene blue staining was used as a loading control. Bottom panel shows the blot for siRNA, which was hybridized with a  $^{32}$ P-labeled transcript corresponding to the 5'-terminal region (400 nt) of *PDS*. Each lane contained 50  $\mu$ g RNA. The arrow indicates the position of a 25-base DNA oligonucleotide.

panel, +) using a *PDS*-specific probe, whereas no *PDS*-related siRNA could be detected in either WT control seedlings (Figure 3f, lower panel, 0) or untreated At-PDSi seedlings (Figure 3f, lower panel, –).

To rule out any toxic or non-specific secondary physiologic effects because of 17 $\beta$ -estradiol-induced *PDS* silencing, T<sub>2</sub> seeds of At-PDSi line 1, which showed a 3 : 1 segregation ratio for Hyg<sup>R</sup>:Hyg<sup>S</sup>, were germinated on an inducer-containing medium without hygromycin, the selective antibiotic. After 3 weeks of incubation, 15 out of 19 T<sub>2</sub> seedlings showed the photobleaching phenotype and ceased to grow (Figure 3e), whereas the remaining four seedlings were normal. These four seedlings were sensitive to hygromycin as they ceased to grow after being transferred to a hygromycin-containing medium. The approximately 3 : 1 segregation pattern of both the selection marker and the photobleaching phenotype suggested that the four hygromycin-sensitive plants were WT, in agreement with the Mendelian segregation ratio for a single transgenic locus. These four plants showed no response to 17 $\beta$ -estradiol and did not exhibit any morphological alteration, indicating that the inducer had no secondary non-specific physiological effect on WT plants. Our results suggest that the inducible RNAi system is able to silence the endogenous *PDS* of *Arabidopsis* at the seed germination stage with comparable efficiency and effectiveness as the constitutive 35S-*PDSi* transgene.

We also examined post-germination induction of *PDS* silencing. Two- or four-week-old T<sub>2</sub> At-PDSi seedlings on the culture medium in the absence of the inducer were transferred to the inductive medium, and similar results were obtained from the seedlings of both age groups. We

observed two photobleaching phenotypes. In the first group which includes At-PDSi lines 2, 5, 7, and 8, a strong *PDS* silencing was seen, 1 week after induction, with newly emerged leaves showing uniform bleaching surrounding the central area of the leaves. The bleaching was subsequently propagated to the entire leaf. Figure 4(a–d) shows results from one representative line 2. Most of these plants with a strong photobleaching phenotype stopped growing. The second group includes At-PDSi lines 1, 3, 4, 6, and 9–12, and these lines showed varying photobleaching phenotypes. After 2 weeks of induction, photobleaching was limited to the areas near the veins (Figure 4e, a plant of line 12) or to the white/green patchy regions in the entire leaf (Figure 4f, a plant of line 1); however, the leaves became near-white over the next 2 weeks (Figure 4g,h, a plant of line 1). Although the *PDS* silencing extended to most rosette leaves and some cauline leaves (Figure 4h), these plants with varying degree of *PDS* silencing could still develop normally and were fertile after transfer to the soil.

We collected seeds from six T<sub>2</sub> plants of At-PDSi line 1 with induced silencing. When germinated on the selective medium (MS + Hyg), none of the T<sub>3</sub> progeny showed *PDS* silencing. When the seeds were germinated on MS medium with neither the selective antibiotic nor the inducer, four T<sub>2</sub> lines showed no *PDS* silencing. On the other hand, more than 10% of the progeny seedlings of the other two T<sub>2</sub> lines showed a constitutive photobleaching phenotype. The constitutive bleached plants presumably derived from some

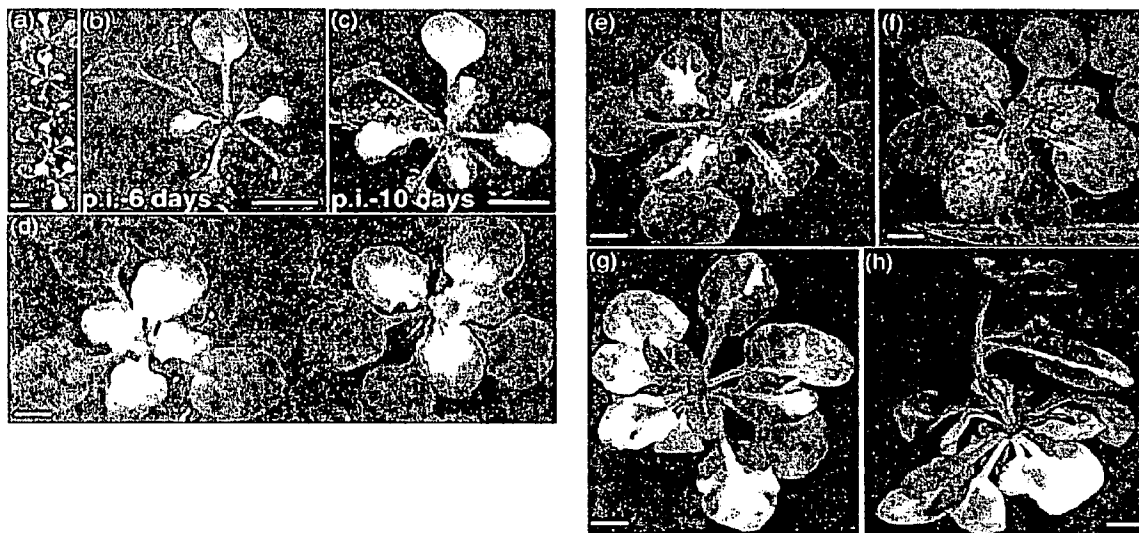


Figure 4. Post-germination induction of *PDS* silencing in At-PDSi *Arabidopsis thaliana* transgenic plants.

(a–d) Strong induction of *PDS* silencing was seen in 2-week-old seedlings of line 2 after transfer to the inductive medium for 6 days (a, b) or 10 days (c). Note that (b) and (c) are from the same plant. Four-week-old seedlings of line 2 were transferred to the inductive medium and the picture was taken at 2 weeks post-induction (d).

(e–h) Delayed onset of *PDS* silencing. Four-week-old seedlings from different At-PDSi transgenic lines 12 (e) and 1 (f–h) were transferred to the inductive medium. Photobleaching was limited to the perivascular regions (e) or white/green patchy regions (f). The plants were photographed at 2 weeks after induction. Photobleaching progressed with time in the old leaves and extended to new rosette and cauline leaves when plants were photographed at 3 weeks (g) or 4 weeks (h) after induction. All scale bars represent 0.5 cm.

converted germ line cells in the L2 layers of  $T_2$  plants that have undergone  $17\beta$ -estradiol-induced *Cre/loxP* DNA recombination (Zuo *et al.*, 2001), and therefore expressed G10-90-*PDSi* constitutively (Figure 1b).

#### Molecular analyses of inducible *PDS* silencing in *At-PDSi* plants

The delayed onset of *PDS* silencing in the second group prompted us to analyze the relationship between the photobleaching phenotype, endogenous *PDS* mRNA levels, and dsRNA induction upon  $17\beta$ -estradiol-induced *Cre/loxP* DNA excision.  $T_2$  progenies of *At-PDSi* line 1 were analyzed in detail. First, dsRNA corresponding to the *PDSi* transcript region (Figure 1) was analyzed. Two-week-old seedlings of line 1-1 (heterozygous) and line 1-2 (homozygous) were transferred to the inductive medium. RNA was extracted from a portion of the seedlings at 42 h post-induction. Total RNA was digested with *Rnase1*<sup>TM</sup> (Promega, USA), and dsRNA was analyzed by hybridization with a *PDS* 5'-terminal probe containing sequences corresponding to the *PDSi* region (Figure 1). Figure 5(b) shows that signals of the expected size were detected in the treated seedlings of both lines 1-1 and 1-2 (lanes 2 and 3), but not in the untreated seedlings of line 1-1 (lane 1).

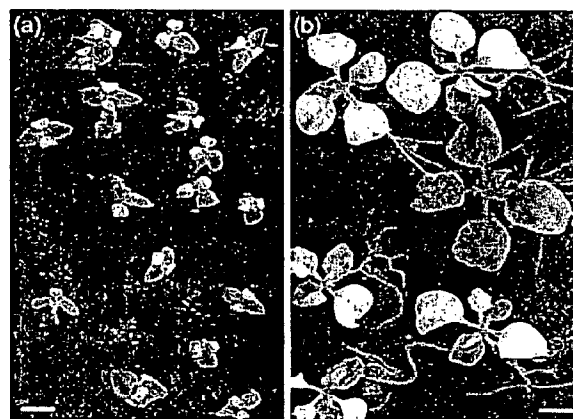
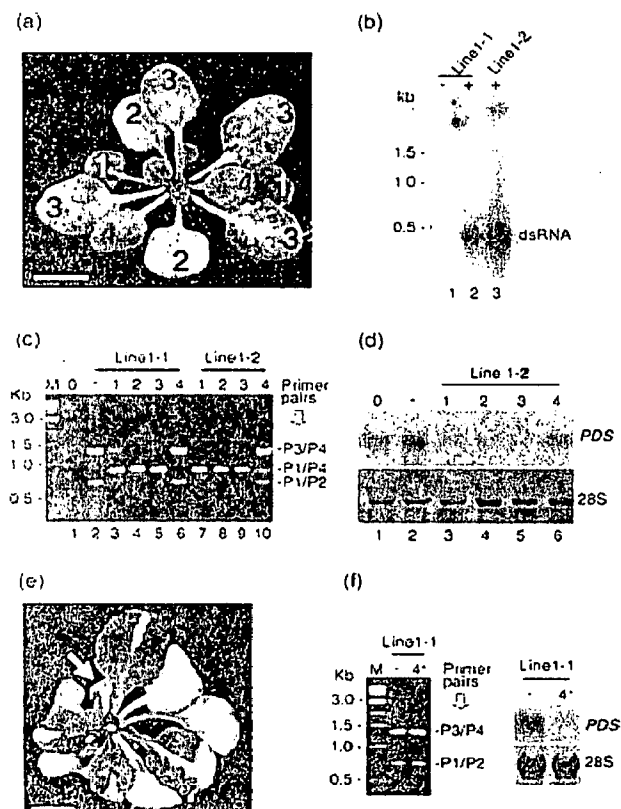


Figure 6. Inducible *PDS* silencing with uniform photobleaching phenotype in one *Nicotiana benthamiana* transgenic (Nb-*PDSi*) line.

(a) Seeds were germinated on the selective medium in the presence of the inducer (MS + Hyg +  $17\beta$ -estradiol), and seedlings were photographed at 2 weeks post-germination.

(b) Two-week-old seedlings were transferred to the inductive medium without hygromycin, and seedlings were photographed at 2 weeks post-induction. The green plant is presumably a WT plant that exhibited no secondary effect upon  $17\beta$ -estradiol treatment. Scale bar = 0.5 cm.

Figure 5. Molecular characterization of delayed onset of *PDS* silencing in *A. thaliana* transgenic plants.

(a) Two-week-old seedlings of *At-PDSi* line 1-1 were transferred to the inductive medium, and the plant was photographed at 18 days post-induction. Scale bar represents 0.5 cm. Leaves with increasing degrees of photobleaching were labeled with numbers 1-4, and those with a similar phenotype were labeled with the same number. Leaves designated 1 were formed before seedlings were transferred to the inductive medium.

(b) Seedlings of *At-PDSi* line 1-1 and 1-2 were either untreated (-) or treated (+) for 42 h with  $17\beta$ -estradiol, RNA was analyzed for the presence of pre-silencing dsRNA. Lane 1, line 1-1, untreated, 100  $\mu$ g RNA; lane 2, treated line 1-1, 50  $\mu$ g RNA; lane 3, treated line 1-2, 100  $\mu$ g RNA. RNAs were digested with *Rnase1*<sup>TM</sup> before being loaded onto formaldehyde-containing gels. The filter was hybridized with a *PDS*-specific 5' sequence corresponding to the *PDSi* region (Figure 1a). Molecular weight standards are shown on the left. (c) PCR analysis of genomic DNA prepared from WT Columbia (lane 1), untreated line 1-1 (lane 2), 18-day  $17\beta$ -estradiol-treated line 1-1 (lanes 3-6) or line 1-2 (lanes 7-10). Leaves with similar phenotype (panel a) were pooled from several plants, and the group numbers indicated on top of the blot. Primers P1-4 were used as illustrated in Figure 1(b). The expected PCR products from different combinations of primer pairs were indicated on the right, M, DNA molecular markers.

(d) Northern analysis of endogenous *PDS* mRNA levels. RNAs were extracted from the same samples as for DNA preparation (panel c), and the corresponding group numbers are indicated on the top. Each lane contained 5  $\mu$ g RNA.

(e) Showing one plant of *At-PDSi* line 1-1 treated with  $17\beta$ -estradiol for 18 days as described in (a) one week after been transferred to high light intensity. Note that the silenced leaves including the old ones (one indicated by arrow) became strongly bleached. Compare this plant to those shown in Figure 4(g,h). Scale bar = 0.5 cm.

(f) Molecular analysis of inducer-treated transgenic plants 1 week after inducer withdrawal and transfer to the soil. Some seedlings of *At-PDSi* line 1-1 treated with  $17\beta$ -estradiol for 18 days as described in (a) were transferred to the soil. After 1 week, leaves belonging to group 4 as well as two younger leaves that displayed photobleaching phenotype were pooled for PCR (left panel) and Northern blot analysis (right panel). Untreated line 1-1 was used as controls (-). For right panel, each leaf contained 5  $\mu$ g RNA.

For the remaining seedlings on the inductive medium, new leaves that emerged after 1-week induction also became patchy in appearance, and photobleaching progressed with time in both the heterozygous  $T_2$  line 1-1 and the homozygous  $T_2$  line 1-2, similar to that in the parental  $T_1$  line 1. To facilitate further analysis, leaves with varying degrees of photobleaching were numbered as shown in Figure 5(a).

PCR analysis was performed using primers specific for the excised sequences and flanking non-excised sequences (see Figure 1b). Based on a previous study (Zuo *et al.*, 2001), the P1/P2 and P3/P4 primer pairs were expected to yield PCR fragments of 696 and 1331 bp, respectively, from a non-recombinant T-DNA. By contrast, following Cre//oxP-mediated recombination and reconstitution of the G10-90-*PDSi* transcription unit, the P1/P4 primer pair produced a PCR product of 992 bp (Figure 1b). Leaves with similar phenotype were pooled from several plants for DNA preparation at 18 days post-induction. No fragment was amplified from WT Columbia (Figure 5c, lane 1), whereas P1/P2 and P3/P4 fragments were detected in untreated line 1-1 (Figure 5c, lane 2), indicating no DNA recombination. The P1/P4 fragment was detected in bleached (group 2 leaves) and patchy leaves (group 3 leaves) of both line 1-1 (Figure 5c, lanes 4 and 5) and line 1-2 (Figure 5c, lanes 8 and 9), indicating complete DNA excision in these leaves after 2 weeks of inducer treatment. In the near-green young leaves (group 4 leaves), however, varying amounts of the DNA excision were found (Figure 5c, lanes 6 and 10). There was no DNA excision in the sample collected from group 4 leaves of line 1-1 because only P1/P2 and P3/P4 fragments were amplified (Figure 5c, lane 6). By contrast, P1/P2, P3/P4, and P1/P4 fragments were detected in the samples collected from group 4 leaves of line 1-2 (Figure 5c, lane 10), indicating that DNA recombination occurred in some of the leaves.

We also found that DNA excision occurred in the old leaves (group 1 leaves) that were already developed before plants were transferred to the inductive medium. P1/P4 fragment was amplified from both line 1-1 (incomplete excision; Figure 5c, lane 3) and line 1-2 (complete excision; Figure 5c, lane 7), consistent with the highly effective inducer-dependent DNA recombination, probably as a result of penetration of 17 $\beta$ -estradiol to almost all cells in the lower leaves.

Endogenous *PDS* mRNA levels of  $T_2$  line 1-2 were assessed by Northern analysis 18 days after induction. Consistent with the photobleaching phenotype and DNA excision, *PDS* mRNA levels were significantly decreased in groups 2 and 3 leaves (Figure 5d, lanes 4 and 5), and slightly decreased in group 4 leaves compared to that in untreated line 1-1 control seedlings (lane 2) and WT Columbia control seedlings (lane 1). As expected, *PDS* mRNA was also degraded in group 1 old leaves (Figure 5d, lane 3),

consistent with the DNA excision assay. Although *PDS* silencing in old leaves was indeed induced after treatment, the persistence of the green color presumably resulted from the continued presence of carotenoids that were synthesized before the  $T_2$  seedlings were transferred to the inductive medium. This residual amount of pre-formed carotenoids was able to protect chlorophylls from photo-oxidation under low light intensity (our assay condition: 35  $\mu\text{mol sec}^{-1} \text{m}^{-2}$ ). However, when these plants were transferred to high light intensity (70  $\mu\text{mol sec}^{-1} \text{m}^{-2}$ ), all leaves including the old ones became strongly bleached (Figure 5e).

Some line 1-1 seedlings treated with the inducer for 18 days were transferred to the soil, and the phenotype of group 4 leaves as well as that of the newly emerged rosette leaves was followed. Group 4 leaves became patchy in appearance, and they became progressively bleached until near-white in appearance in 1 week. Similar results were observed in another two newly grown rosette leaves, which displayed limited areas of photobleaching. DNA and RNA were extracted from new photobleached leaves (including group 4 leaves), and PCR analysis showed that only P1/P2 and P3/P4 fragments were amplified (Figure 5f), indicating no DNA excision in these leaves. However, RNA analysis demonstrated a severe reduction in *PDS* mRNA levels, indicating that the *PDS* gene was silenced presumably via signals generated by the lower leaves.

#### *Inducible silencing of endogenous PDS gene in transgenic Nicotiana benthamiana*

Similar results were obtained in *N. benthamiana* transformed with the pX7-*PDSi*(Nb) construct. Inducible *PDS* silencing was observed when the inducer was given at germination (Figure 6a) or post-germination stages (Figure 6b). Northern analysis of endogenous *PDS* mRNA levels (data not shown) confirmed the visual photobleaching phenotype, suggesting that the inducible RNAi system is capable of inducing endogenous gene silencing in *N. benthamiana* and probably other plant species as well.

#### Discussion

In this work, we report the development of an inducible dsRNA-mediated silencing (inducible RNAi) system for conditional gene silencing in transgenic plants. This system contains two steps: (i) the inducible expression of dsRNA resulting from 17 $\beta$ -estradiol-induced DNA recombination and (ii) induction of target gene silencing by the dsRNA. A detailed characterization of the 17 $\beta$ -estradiol-inducible silencing system in transgenic *A. thaliana* and *N. benthamiana* plants demonstrated that this system is stringently controlled and can produce, at high efficiency, conditional silencing of both a *GFP* transgene and an



endogenous *PDS* gene and without any detectable secondary affect. At seed germination stage, all tested At-PDSi lines showed a uniform photobleaching phenotype similar to that obtained with constitutively silenced 35S-PDSi lines. This suggests that the efficiency and effectiveness of the inducible RNAi system against endogenous genes are comparable to those obtained with constitutive expression of dsRNA (constitutive RNAi).

The highly efficient gene silencing obtained by intron-containing dsRNA expressed from a constitutive promoter would produce loss-of-function transgenic plants similar to null mutants. If the target gene is essential for basic cell function and development, constitutive RNAi would probably prevent shoot regeneration, cause plant lethality or embryogenesis defect, and block the ability of the transformed plants to produce subsequent generations. These problems can be avoided by using the inducible RNAi system described here. Moreover, the inducible RNAi system provides the possibility to induce gene silencing at different stages of plant development post-germination. This is demonstrated by the At-PDSi lines described here, in which a portion of the tested 2- or 4-week-old seedlings showed strong photobleaching after induction similar to that shown by transgenic plants with 35S-PDSi phenotypes. Moreover, the varying initiation and degrees of inducible gene silencing generate plants with a range of loss-of-function phenotype much like an allelic series, facilitating functional analysis.

Both heterozygous (line 1-1) and homozygous (line 1-2) progeny of At-PDSi line 1 retain the ability to reproduce inducible *PDS* silencing as the parental line, indicating that the inducible RNAi can be transmitted to the next generation. Having stable and reproducible RNAi transgenic lines would allow genetic crosses to be made and investigations of gene functions to be carried out with subsequent generations. Other silencing induction systems, such as *Agrobacterium* infiltration (Voinnet and Baulcombe, 1997) and infection with vectors derived from RNA viruses (Dalmay et al., 2000; Ratcliff et al., 2001) or DNA viruses (Peele et al., 2001; Turnage et al., 2002), have limited utility for functional genomics because of their transient nature, and the silencing effects are not heritable. Moreover, *Agrobacterium* infiltration cannot be applied to *Arabidopsis* because of its small plant size and clumpy rosette leaves. In the case of virus-induced gene silencing, some infected plants display stunted growth and they often do not produce inflorescences, flowers, or seeds (Turnage et al., 2002). Moreover, disease symptoms caused by viral infection could confound the interpretation of the phenotype as a result of silencing of the target gene. By contrast, the inducible RNAi system described here can be triggered by simply treating plants with the inducer for a certain period of time. Treated plants display a specific silencing phenotype without any non-specific effects (see Figures 2a, 4e-h and 6b).

We have chosen the inducible Cre/loxP DNA excision system (CLX) rather than the XVE transient inducible system (Zuo et al., 2000) to produce the inducible RNAi for several reasons. The mechanism for RNA silencing involves an initial induction process followed by a systemic spread of the silencing signal (for review, see Mlotshwa et al., 2002). The local initiation of gene silencing by dsRNA is equally effective for both transgene and endogenous genes (Smith et al., 2000; Wesley et al., 2001). However, unlike the widespread, persistent silencing observed for a *GFP* transgene, systemic silencing of endogenous genes was transient and limited (Palauqui and Vaucheret, 1998; Voinnet et al., 2000). Amplification of the signal is necessary for efficient systemic silencing (Palauqui and Vaucheret, 1998; Voinnet et al., 1998). It has been proposed that the silencing signal is perpetuated by transgenes, but not by endogenous genes (Fagard and Vaucheret, 2000; Mlotshwa et al., 2002). The CLX system can rescue the weak amplification of the silencing signal by endogenous genes. Once the Cre/loxP-mediated DNA excision occurred upon inducer treatment, expression of the downstream intron-containing dsRNA would be permanently activated. This situation mimics the expression of an RNAi using a constitutive promoter, which is the most efficient, effective, and high-throughput system for gene silencing, and the systemic spread of silencing signal is therefore not required. Expression of dsRNA from the inducible XVE system (Zuo et al., 2000) without DNA recombination would require repeated applications of 17 $\beta$ -estradiol to the transgenic plants for sustained expression, and this may not be practical for plants growing in the soil.

The strong photobleaching induction in At-PDSi lines (lines 2, 5, 7, and 8) at post-germination stages is believed to be a result of complete DNA excision, which would reconstitute the G10-90-*PDSi* transcription unit to produce *PDSi* transcripts constitutively throughout the entire plant. In this case, no silencing signal amplification was required. This interpretation is consistent with our PCR analysis of At-PDSi lines (second group), displaying a delayed onset of gene silencing. In both line 1-1 and line 1-2 leaves, complete excision of DNA within the loxP sites showed strong photobleaching or patchy *PDS*-silencing phenotype. In near-green leaves where incomplete or no DNA excision was detected, only a weak systemic spread (weak signal amplification) of *PDS* silencing to upper rosette leaves was seen during the 18-day incubation with the inducer (Figure 5a-d). However, upon an additional week of growth after inducer withdrawal, strong *PDS* gene silencing was also detected in group 4 leaves and in the next two younger leaves (Figure 5f). These results suggest a limited systemic translocation of the silencing signal to two to three upper leaves.

Because of 17 $\beta$ -estradiol instability (Zuo et al., 2000), a second or even multiple treatment with fresh inducer may

be needed to fully reactivate this RNAi system in some transgenic lines. With appropriate improvement of the induction conditions, a higher DNA excision efficiency, and therefore a higher proportion of lines showing strong induction, may be obtained. Nevertheless, the incomplete *Cre/loxP* DNA excision, which results in genetic chimera in transgenic plants, may provide a useful system to study mechanisms of long-distance signal transduction in gene silencing in *Arabidopsis*, which is difficult to graft (Turnbull *et al.*, 2002). The mechanisms involved in systemic RNA silencing in plant systems are being actively investigated using grafting and transient expression approaches with *N. benthamiana* or *N. tabacum* (Guo and Ding, 2002; Mallory *et al.*, 2001; Voinnet *et al.*, 2000). No mutations specific to systemic silencing have yet been reported in plant systems. Because of the small plant size and the clumpy rosette leaves, it is impossible to carry out localized infiltration of *Arabidopsis* with *Agrobacterium*, and grafting manipulation in *Arabidopsis* is also a challenging task. For these reasons, the ability to generate genetic chimera in transgenic *Arabidopsis* producing RNAi only from treated tissues would be very useful for future investigations. As shown by PCR analysis in At-PDSi line 1-1 and line 1-2, DNA excision occurred in lower rosette leaves, but not in upper rosette leaves. Gene silencing resulting from local RNAi induction (complete excision, Figure 5c,d) or signal-mediated long-distance (no excision) induction (Figure 5f) can be predicted by simple PCR analysis. The ability to generate genetic chimera in *Arabidopsis* may also find useful applications in research on other types of long-distance signaling (e.g. flowering time) in plants.

## Experimental procedures

### Plasmid construction

DNA manipulations and cloning were carried out using standard procedures (Sambrook *et al.*, 1989). The third intron of *Arabidopsis* actin gene 11 (ATU27981, nt 1957–2111) was selected for the intron-containing intermediate construct (pSK-int). This intron was amplified by PCR using two primers: Pint5', 5'-TACGTAAGTA-GATCTTCAACACC-3'; and Pint3', 5'-GGAATTCTGCAAACACACA-AGACAAT-3'. The primers were designed such that their border sequences contained the consensus sequence (bold letters) for plant introns: AG//GTAAGT...TGCAG//G (Shapiro and Senapathy, 1987). Two restriction sites *Sna*BI and *Eco*RI (underlined) were added for cloning purposes. A PCR fragment of 155 bp was digested with *Sna*BI/*Eco*RI and cloned into *Eco*RV/*Eco*RI-digested pBluscript II SK+ to yield the intermediate construct pSK-int (Figure 1a).

To clone sequences encoding the inverted-repeat RNA into the pSK-int intermediate vector, a 357 bp fragment corresponding to nucleotides (nt) 360–716 of the *GFP* 3'-terminal sequence (Voinnet and Baulcombe, 1997) was cloned into the 5' and 3' arms of the intron (Figure 1a), and the resulting plasmid was named as pSK-GFPi. The 5'-terminal sequences of *PDS* of *A. thaliana* and *N. benthamiana* were obtained by RT-PCR amplification with

specific primers. For *A. thaliana* *PDS*, the primers were: Pat5', 5'-GACTAGTATGGTTGTGTTTGGGAATG-3'; and Pat3', 5'-GATATCCTTCCATGCAGCTATC-3'. These primers were used to obtain a fragment of 405 bp corresponding to nt 128–532 of the *A. thaliana* *PDS* cDNA (L16237), and *Spe*I and *Eco*RV restriction sites (underlined) were added to the cDNA fragment. For PCR amplification of the *N. benthamiana* *PDS* sequence, the primers were: Pnb5', 5'-GACTAGTATGCCTCAAATTGGACTTGT-3'; and Pnb3', 5'-CAGCTG-TAGACAAACCACCCAAAC-3' homologous to regions of the tomato *PDS* cDNA (M88683) nt 318–337 and nt 676–696, respectively. These primers were designed with the addition of *Spe*I and *Pvu*II restriction sites (underlined). Using *N. benthamiana* RNA as templates, a 386 bp fragment was obtained with RT-PCR, whose sequence exhibited high homology with the tomato *PDS* cDNA. RT-PCR fragments derived from *A. thaliana* (At) and *N. benthamiana* (Nb) were cloned into the pCR-Blunt vector (Invitrogen, USA) to give pCR-PDS(At) and pCR-PDS(Nb), respectively. Fragments of *Spe*I-*Eco*RI and *Hind*III-*Xho*I were inserted into both arms of the intron of pSK-int digested with the appropriate restriction enzymes as shown in Figure 1(a) to obtain pSK-PDSi(At) and pSK-PDSi(Nb), respectively.

For inducible dsRNA transformation constructs, the kanamycin-resistance gene in pX6-GFP (Zuo *et al.*, 2001) was replaced with a hygromycin-resistance gene, and the derivative called pX7-GFP. To create an inducible expression of intron-containing dsRNA, fragments of *Xho*I-*Xba*I from pSK-GFPi, pSK-PDSi(At), and pSK-PDSi(Nb) were subcloned into pX7-GFP digested with *Xho*I/*Spe*I (*Xba*I and *Spe*I are compatible), resulting in pX7-GFPi, pX7-PDSi(At), and pX7-PDSi(Nb), respectively (Figure 1b).

In addition, the *Pst*I-*Sac*I fragment from pSK-PDSi(At) was cloned into a modified binary vector pCambia-1300 (AF234296), which contained a 35S promoter and a 35S terminator, to give pCambia-PDSi(At), which is a constitutive RNAi construct (Figure 1c).

Upon request, vectors described in this paper are available to academic researchers for non-commercial projects.

### Plant materials, transformation, and growth conditions

A transgenic *N. benthamiana* line (GFP-16c) carrying a 35S-GFP transgene with a kanamycin-selectable marker at a single locus in homozygous condition (Ruiz *et al.*, 1998) and a transgenic *A. thaliana* ecotype C24 line (Dalmay *et al.*, 2000) carrying a similar transgene were used for pX7-GFPi transformation. *A. thaliana* ecotype Columbia was used for pX7-PDSi(At) and pCambia-PDSi(At) transformation. WT *N. benthamiana* was used for pX7-PDSi(Nb) transformation. *N. benthamiana* transformation was carried out by co-culture with *Agrobacterium*, whereas *A. thaliana* was transformed by the floral dip method (Clough and Bent, 1998). The selective medium contained MS medium plus hygromycin (20 mg l<sup>-1</sup> for *A. thaliana* and 40 mg l<sup>-1</sup> for *N. benthamiana*), whereas the inductive medium contained, in addition, 17 $\beta$ -estradiol (2  $\mu$ M). GFP fluorescence was examined using a 100 W hand-held long-wavelength UV lamp.

### Analyses of RNA and DNA

Total RNA was isolated from plant tissues by LiCl precipitation (Verwoerd *et al.*, 1989). The LiCl supernatant fraction was precipitated with 3 volumes of ethanol to obtain genomic DNA and low molecular weight RNA (siRNA). dsRNA was obtained by digesting total RNA with Rnase1<sup>TM</sup> (Promega, USA) (0.5 U Rnase  $\mu$ g<sup>-1</sup> total RNA) at 37°C for 3 h. For Northern analysis, total RNA or dsRNA was separated on 1.2% agarose formaldehyde gels, transferred to

Hybond-N+ membranes, and hybridized with <sup>32</sup>P-labeled cDNA probes specific for the respective RNA. Low molecular weight RNA analysis was done as described (Hamilton and Baulcombe, 1999; Llave *et al.*, 2000). The probes for GFP and PDS siRNA were <sup>32</sup>P-labeled 3'-terminal 356 nt of GFP or 5'-terminal 400 nt of PDS antisense RNA, respectively, transcribed by T7 RNA polymerase. The PCR analysis with approximately 200 ng of genomic DNA was subjected to 94°C for 20 sec, 50°C for 20 sec, and 72°C for 2 min for 30 cycles. Primers for PCR analysis were: P1, 5'-GCCGCCACG-TGCCGCCACGTGCCGCC-3'; P2, 5'-CTCGTCAATTCCAAGGGCAT-CGGT-3'; P3, 5'-CTGGACACAGTGCCCGTGTCCGA-3'; P4, identical to P1 for intron amplification (see Results).

### Acknowledgements

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## TECHNICAL ADVANCE

# Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA

Shuai Chen, Daniel Hofius, Uwe Sonnewald and Frederik Börnke\*

Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Corrensstrasse 3, 06466 Gatersleben, Germany

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\*For correspondence (fax +49 39482 5515; e-mail boernke@ipk-gatersleben.de).

## Summary

Downregulation of endogenous genes via post-transcriptional gene silencing (PTGS) is a key to the characterization of gene function in plants. The recent discovery that double-stranded RNA (dsRNA) is an extremely effective trigger of gene silencing greatly enhanced the predictability of this approach. However, strong constitutive silencing often leads to pleiotropic effects, which make it difficult to directly relate phenotype to gene function, or even interferes with the recovery of viable transgenic plants. Here, we show that strong genetic interference can be achieved in a chemically inducible fashion, allowing for temporal and spatial control of gene silencing in transgenic plants. To this end, transgenic tobacco plants were established expressing dsRNA in the form of intron-spliced hairpin structures under the control of the ethanol-inducible *alc* gene expression system. Targeting magnesium (Mg)-chelataase subunit I (*Chl I*) and glutamate 1-semialdehyde aminotransferase (*GSA*), both involved in chlorophyll (chl) biosynthesis, resulted in rapid and specific mRNA degradation upon induction with ethanol. Ethanol-inducible silencing of the target genes caused strong but transient phenotypical alterations featured by a progressive loss of chl in young leaves, which persisted for about 7–9 days before newly growing leaves completely recovered. About 10–30% of the primary transformants showed phenotype development upon induction. Local silencing of *Chl I* could be achieved by confined ethanol treatment of a single leaf without affecting any other part of the plant. Inducible gene silencing using the *alc* system promises to obviate the problems associated with constitutive RNA silencing and enables to dissect primary and secondary effects of PTGS at temporal and spatial resolution.

**Keywords:** chemically inducible expression, ethanol, double-stranded RNA, gene silencing, functional genomics, *Nicotiana tabacum*.

## Introduction

The recent completion of the *Arabidopsis thaliana* genome sequence (The Arabidopsis Genome Initiative, 2000) and the accumulation of sequence data from a number of other model plants and important crop species, such as rice (Goff *et al.*, 2002; Yu *et al.*, 2002), have provided a vast new resource to define gene function at the morphological, biochemical, or physiological level. A critical step in exploiting these genomic resources, however, depends on the development of novel tools and approaches for the functional analysis of a given gene. In the past, the use of loss-of-function or reduced-expression mutants has proven to be a powerful tool to relate a mutant phenotype to the function of a particular gene. Traditionally, these reverse

genetic approaches rely on transposon insertions or classic genetic screens (for review, see Page and Grossniklaus, 2002). However, their use is limited by the untargeted nature of the mutagenesis and their restriction to a few genetically tractable plant species. The discovery of the antisense phenomenon of plant gene silencing (van der Krol *et al.*, 1988; Smith *et al.*, 1988), and subsequently co-suppression (van der Krol *et al.*, 1990; Napoli *et al.*, 1990), provided an alternative means for investigating the role of specific gene products in plant growth and metabolism and has been particularly versatile in studying plant primary metabolism (Frommer and Sonnewald, 1995; Stitt and Sonnewald, 1995). The antisense and co-suppression

phenomena are collectively referred to as post-transcriptional gene silencing (PTGS), which describes a nucleotide sequence-specific RNA degradation process naturally providing a defense mechanism against invasive nucleic acids such as viruses, transposons, and transgenes (Baulcombe, 2002; Matzke *et al.*, 2001; Vance and Vaucheret, 2001; Voinnet *et al.*, 1998). A set of mechanistically related pathways were also found in fungi (quelling) and in animals (RNA interference (RNAi)) and requires a conserved set of gene products (for recent reviews, see Carthew, 2001; Cogoni and Macino, 2000; Plasterk, 2002). According to current models of PTGS, an endogenous RNA-dependent RNA polymerase initially synthesizes a double-stranded RNA (dsRNA) molecule using the target transcript as template (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000), which is subsequently processed into 21–25 ntRNA fragments of both polarities (Hamilton and Baulcombe, 1999). These short interfering RNAs (siRNAs) are then incorporated into a dsRNA-induced silencing complex (RISC) to guide cycles of specific RNA degradation (Hammond *et al.*, 2000; Tang *et al.*, 2003; Zamore *et al.*, 2000). In some experiments, however, when conventional antisense and co-suppression constructs are used to trigger PTGS, RNA-silencing occurs only in a portion of the transformants or their progenies or the constructs even fail to induce silencing, rendering these approaches largely ineffective. Recently, considerable progress in effectively triggering PTGS has been made by using constructs designed to express dsRNA fragments, usually in the form of self-complementary hairpin RNA, consistently yielding a high degree and frequency of PTGS. This often gives rise to phenotypes that resemble those of the null alleles of the target genes (Chuang and Meyerowitz, 2000; Levin *et al.*, 2000; Smith *et al.*, 2000). However, because of the high degree of silencing, which is achieved by this method, functional analysis of a particular gene product required during plant transformation and regeneration from culture is limited, as viable plants might not be recovered. Moreover, constitutive gene silencing consistently entails pleiotropic effects, which might superimpose the primary impact of reduced gene expression and thus mask true gene function. This is especially true, if metabolic processes are under scrutiny, as the observed phenotype could rather reflect a physiological adaptation to the loss of a metabolic function than a primary response to the latter. To overcome these problems a system for inducible gene silencing of candidate genes would be highly desirable.

The problems have partially been solved by the introduction of virus-induced gene silencing (VIGS) systems where a recombinant virus carrying a partial sequence of a host gene is used to infect the plant. When the virus spreads systemically, the endogenous transcripts, which are homologous to the insert in the viral vector, are degraded by PTGS (Baulcombe, 1999). Although several VIGS vectors have been described (Gosselé *et al.*, 2002; Holzberg *et al.*,

2002; Liu *et al.*, 2002; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002), these approaches suffer from a number of shortcomings. First, the use of each system is restricted by the host range of the virus it is derived from and thus no such system is broadly applicable to a wide range of plant species. Second, the VIGS phenotype is superimposed, and sometimes complicated, by at least mild disease symptoms of virus infection, which cause significant biochemical perturbations not directly linked to the suppression of the target gene. This causes problems especially in physiological investigations. Most of these limitations should be overcome by the use of a chemically inducible promoter to drive expression of the silencing construct, allowing the investigator to control when a specific gene will be inactivated. The optimal system would employ a non-toxic inducer with high specificity and minimal potential to elicit physiological responses. Moreover, the system should have the capacity to achieve high level of expression but with a concomitant negligible activity in the absence of the inducer. One such system is the *alc* gene switch based on a regulon derived from the filamentous fungus *Aspergillus nidulans* (Caddick *et al.*, 1998; Roslan *et al.*, 2001; Salter *et al.*, 1998). In plants, the system basically consists of two modules: the AlcR transcriptional regulator expressed from the cauliflower mosaic virus (CaMV) 35S promoter and a modified *alcA* promoter in front of the gene of interest. In the presence of ethanol, AlcR binds to the modified *alcA* promoter and drives expression of the target gene. Several studies demonstrated the efficiency of the *alc* system in a wide range of plant hosts, including *Arabidopsis*, *Brassica napus*, *Nicotiana tabacum*, and potato tubers (Caddick *et al.*, 1998; Junker *et al.*, 2003; Roslan *et al.*, 2001; Salter *et al.*, 1998; Sweetman *et al.*, 2002). Induction of the system can be achieved by either root drenching of the plants with ethanol solution (Caddick *et al.*, 1998; Roslan *et al.*, 2001; Salter *et al.*, 1998) or exposing the plants to ethanol vapor (Sweetman *et al.*, 2002). Alternatively, the *alc* gene switch can also be activated by other related chemicals. A recent study demonstrated a more rapid induction of the system upon the application of acetaldehyde, the physiological inducer of the *alc* regulon in *A. nidulans*, than that of ethanol (Junker *et al.*, 2003).

We initiated the study described herein to determine whether the *alc* system can be used to achieve inducible gene silencing in transgenic tobacco. To this end, nuclear genes involved in the chlorophyll (chl) biosynthetic pathway were targeted, because it was assumed that the host gene silencing would be easy to visualize and quantify as loss of chl. Our data demonstrate that transgenic tobacco plants designed to express double-stranded hairpin constructs under the control of the ethanol responsive promoter exhibit a rapid but transient development of the characteristic phenotype upon induction. Using repeated application of ethanol, extended periods of gene silencing

could be maintained. We further show that spatial silencing of a target gene could be achieved by ethanol treatment of a single tobacco leaf. With these attributes, inducible PTGS using the *alc* system promises to extend the kind of silencing studies that can be carried out in transgenic plants, in particular, with respect to temporal and spatial resolution of silencing effects, rendering the system extremely useful for metabolic studies.

## Results

### Construction of silencing vectors and plant transformation

To evaluate the suitability of the *alc* system for inducible gene silencing, two nuclear target genes were selected, which have previously been described to yield a readily discernable phenotype in conventional antisense experiments. Magnesium (Mg)-chelatase is a heteromeric enzyme complex composed of three-subunits (designated CHL I, CHL H, and CHL D) that catalyzes the incorporation of  $Mg^{2+}$  into protophorphyrin IX, which represents the first committed step in chl biosynthesis. Antisense suppression of *Chl I* in transgenic tobacco led to a strongly reduced green pigmentation as a result of the decreased chl biosynthetic capacity (Papenbrock *et al.*, 2000). A key regulatory step in tetrapyrrole biosynthesis in higher plants, providing the precursors for chl and heme synthesis, is the formation of 5-aminolevulinic acid catalyzed by the activity of glutamate 1-semialdehyde aminotransferase (GSA). Expression of GSA antisense RNA in tobacco plants results in a decline in chl content apparently leading to pale leaves (Höfgen *et al.*, 1994).

Constructs for inducible expression of dsRNA in transgenic plants were assembled in the appropriate plant transformation vector (Caddick *et al.*, 1998), which contained the *AlcR* gene driven by the constitutive CaMV 35S promoter and gene-specific fragments in sense and antisense orientation interspersed by a short intron under control of the modified *alcA* promoter (*alc-dsRNA* gene cassette, Figure 1). In parallel, inducible antisense constructs were made to target *Chl I* and *GSA*, respectively, by putting the corresponding antisense fragment under the control of the ethanol-inducible promoter (*alc-anti* gene cassette, Figure 1). After *Agrobacterium*-mediated gene transfer (Rosahl *et al.*, 1987), 80 primary transformants for each construct were transferred to the greenhouse. Prior to the application of ethanol, all transgenic plants were indistinguishable from wild-type plants, indicating that the promoter was not leaky. Northern analysis of transgene-specific dsRNA further confirmed tight control of the promoter under un-induced conditions in that no detectable levels of dsRNA were present prior to the application of the

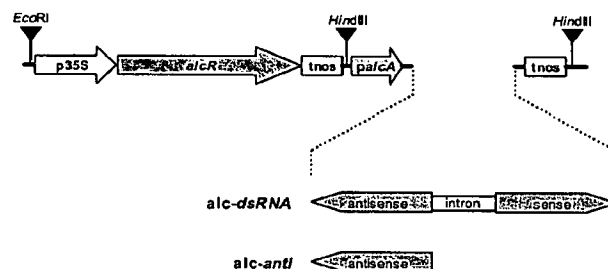


Figure 1. Diagrammatic representation (not to scale) of *alc*-system-derived silencing constructs.

The *alcR* cDNA is under control of the CaMV 35S (*p35S*) promoter and positioned upstream of the *nos* terminator (*tnos*). The *alc-dsRNA* construct contains either a *Chl I* or *GSA* fragment in antisense and sense orientation separated by intron 1 of potato GA20 oxidase (200 bp) in spliceable orientation. The *alc-antisense* construct comprises simply the respective antisense fragment. In both constructs, all fragments were inserted downstream of the chimeric *palcA* promoter, which consists of the CaMV 35S minimal promoter (−31 to +5) fused at the TATA-box to upstream promoter sequences of *alcA* (Caddick *et al.*, 1998).

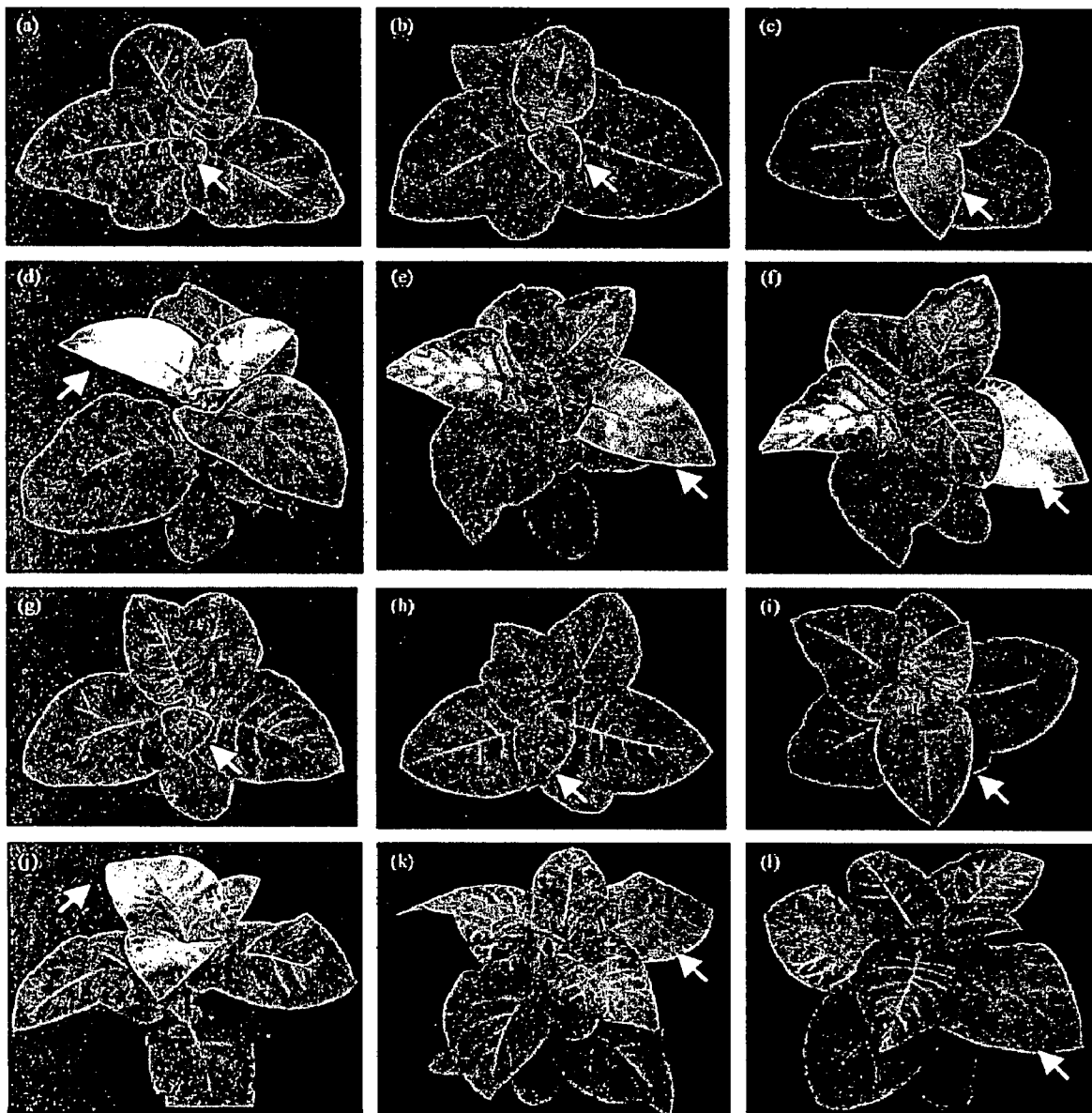
inducer (data not shown). However, after ethanol induction, 26 (33% efficiency) of the *alc-dsChl I* transgenics and 10 (13% efficiency) of the *alc-dsGSA* plants displayed the characteristic phenotype previously described from constitutively silenced plants. Phenotypes of individual transformants varied from heavily bleached leaves with only small green areas remaining (11 *alc-dsChl I* and 4 *alc-dsGSA* plants, respectively) to variegation patterns of likewise green and yellow to white patches (six *alc-dsChl I* and three *alc-dsGSA* plants, respectively) and pale areas along the major leaf veins (nine *alc-dsChl I* and three *alc-dsGSA* plants, respectively). The overall efficiency using the *alc* system for inducible gene silencing was much lower than that of the recently published inducible RNAi system based on estradiol induction (Guo *et al.*, 2003). Whether this was because of differences in constructs or vectors or because of a species difference is currently unknown.

Specific silencing of the target gene in phenotypically affected plants was further confirmed by Northern blotting using gene-specific probes (data not shown). Reduction in *Chl I* and *GSA* mRNA steady state level correlated with the strength of the observed phenotype. Contrastingly, none of the plants solely expressing the antisense fragment targeted against *Chl I* and *GSA* displayed any visible phenotype upon induction (data not shown). Northern blotting was applied to screen for individuals expressing the respective antisense transgenes. Several plants expressing considerable amounts of the antisense transcript could be identified for each construct (data not shown). From these data, we conclude that ethanol-inducible expression of dsRNA causes silencing of endogenous genes comparable to that in constitutively silenced plants. Selected primary transformants for each construct were selfed and the T1 generation was subject to a detailed analysis.

*Rapid and reversible phenotypical changes in plants containing alc-dsRNA expression cassettes*

Three *alc-dsChl I* lines (14, 16, and 45), and two *alc-dsGSA* lines (60 and 70) were chosen for a detailed analysis according to their strong inducible phenotype observed in the  $T_0$  generation. Seeds were germinated on kanamycin-containing medium and resistant seedlings were analyzed 4 weeks

after transfer to soil. Application of 1% ethanol by root drenching led to the development of the characteristic phenotype featured by the loss of chl. The phenotype started to develop approximately 36 h post-induction (hpi) for *alc-dsChl I* lines and approximately 48 hpi for *alc-dsGSA* lines, and was first visible in the top leaves (Figure 2). The bleached patches expanded as the leaves grew, and also appeared in nascent leaves over a period of



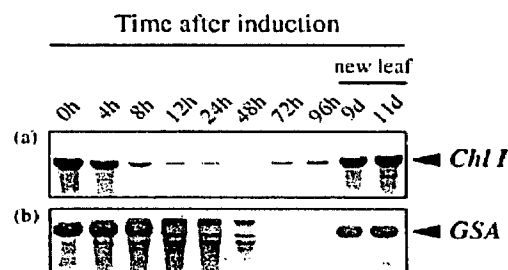
**Figure 2.** Time course of phenotype development in *alc-dsRNA* lines after application of ethanol. (a–f) *alc-dsChl I* line 16 before, and 2, 4, 5, 9, and 12 dpi, respectively. (g–l) *alc-dsGSA* line 60, before, and 2, 4, 5, 7, and 9 dpi. The arrow indicates the type of leaf that has been followed for molecular analyses. Plants (42 days old) were induced via root drenching with 100 ml 1% (v/v) ethanol and photographs were taken at different time points.

7–9 days. After that, newly emerging leaves looked like the wild type again while those initially affected never recovered. Whether this was because of the developmental control of chl-synthesizing enzymes (Härtel *et al.*, 1997; He *et al.*, 1994) or because of stable silencing is currently unknown. Leaves that were mature prior to induction always remained green. Upon a second induction, the silencing phenotype could be re-established in the newly grown leaves of transgenic plants (data not shown).

In some cases, the silencing effect caused by a single induction might be too transient to reveal the full sequence of consequences of reduced gene expression. In order to investigate whether extended periods of gene silencing could be achieved by repeated ethanol treatments, *alc-dsChl I* plants were root-drenched with 1% ethanol every 2 days for 15 days in total. Phenotypic changes became apparent 2 days post-induction (dpi) but, in contrast to the single-induction experiments, they were maintained over the entire period of ethanol treatment (Figure 6b). Therefore, using re-iterated treatments, extended periods of gene silencing can be maintained.

#### Kinetics of inducible gene silencing

In order to follow the kinetics of target transcript reduction in *alc-dsRNA* lines, samples were taken from leaves expected to become phenotypically affected at different time points after application of ethanol. To compensate for plant-to-plant variation, at least three plants were probed at each time point and samples were pooled after preparation of total RNA. Northern analysis revealed that the *Chl I* mRNA was significantly reduced as early as 8 h after ethanol induction. Transcript levels further declined to undetectable levels at 48 hpi so as to rise again from 72 hpi onwards. The initial amount was reached again at approximately 9 dpi in newly emerging leaves, which were phenotypically normal (Figure 3a). *Chl I* transcript levels in ethanol-treated wild-type plants remained stable over the time course of the experiment (data not shown). Similar kinetics of target transcript decay and duration of mRNA downregulation was observed in the *alc-dsGSA* plants (Figure 3b). In the line under investigation (line 60), an additional band migrating above the endogenous *GSA* transcript appeared upon induction and was detectable upto 48 hpi. As PTGS has been shown to affect RNA processing (Mishra and Hanada, 1998), we used RT-PCR to investigate whether splicing of the endogenous *GSA* messenger was impaired in these plants. No PCR product that could possibly represent an incompletely spliced RNA was detected. However, if primers specific for the intron included in the transgene construct were used, a weak but specific band was amplified (data not shown). Therefore, the additional band on the *GSA* Northern blot was tenta-

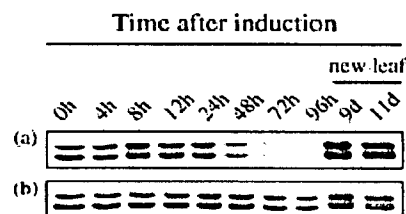


**Figure 3.** Efficient downregulation of target mRNA following ethanol-inducible expression of dsRNA constructs.

(a) Time course of *Chl I* transcript degradation in *alc-dsChl I* plants (line 16). (b) Time course of *GSA* mRNA degradation in *alc-dsGSA* plants (line 60). Transgenic tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being c. 5 cm in size at the time point of induction. After the indicated periods of time, total RNA was isolated and Northern blot hybridization was performed with the respective cDNA probe. At 9 and at 11 dpi, newly grown leaves were taken for the analysis.

tively assigned to the unspliced intron-containing inverted-repeat fragment. This band was never observed in any other *alc-dsGSA* line; however, line 60 had the strongest phenotype and high expression of the transgene might interfere with its correct splicing because of sub-optimal splice sites.

In order to follow the silencing process in *alc-dsGSA* lines on the protein level, samples were taken from the same leaves as before and subjected to a Western analysis using *GSA*-specific antibodies (kindly provided by Dr B. Grimm, Humboldt University, Berlin, Germany). Within 48 hpi, a considerable reduction of the *GSA* amount occurred in the transgenics (Figure 4), which is in good accordance with phenotype development in these plants. No detectable protein was observed 72–96 hpi. When newly developing leaves were probed for *GSA* 9 dpi, the amounts were comparable to those before induction.



**Figure 4.** Time course of *GSA* protein degradation in *alc-dsGSA* plants after ethanol treatment.

(a) Level of *GSA* protein in *alc-dsGSA* plants (line 60) after induction. (b) Ethanol-treated control plants. Plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being c. 5 cm in size at the time point of induction. After the indicated periods of time, total protein was prepared and Western blot analysis was performed using an anti-*GSA* antibody. At 9 and at 11 dpi, newly grown leaves were taken for the analysis.



### Changes in chlorophyll contents upon induction of gene silencing

In order to investigate the degree of gene silencing, which could be achieved with the ethanol-inducible system compared to plants constitutively silenced for *GSA* and *Chl I* (Höfgen *et al.*, 1994; Papenbrock *et al.*, 2000), the decrease in chl content in phenotypically affected leaves over time was taken as an indirect measure for chl biosynthetic capacity. Therefore, leaves were followed that were approximately 5 cm in length at the time point of induction. Samples were taken at different time points and analyzed for their chl content. To compensate for local variations within a plant, two samples were taken from each leaf. To compensate for plant-to-plant variation, at least five plants were harvested at each time point for each construct tested. After application of ethanol, a gradual decline in chl content was observed in lines expressing dsRNA constructs (Figure 5), whereas chl content in ethanol-treated control plants increased over the time course of the experiment as a result of the developmental control of chl biosynthesis. In case of *alc-dsChl I* plants, the progressive loss of chl became apparent as early as 24 hpi, while ethanol-treated *alc-dsGSA* plants lost their chl more slowly. This is in good accordance with the temporal differences in phenotype development of the two mutants. Seven days post-induction, chl content in *alc-dsChl I* plants was only approximately 3% of that before treatment, whereas chl content in phenotypically affected leaves of *alc-dsGSA* was reduced to levels of about 20% of those before treatment.

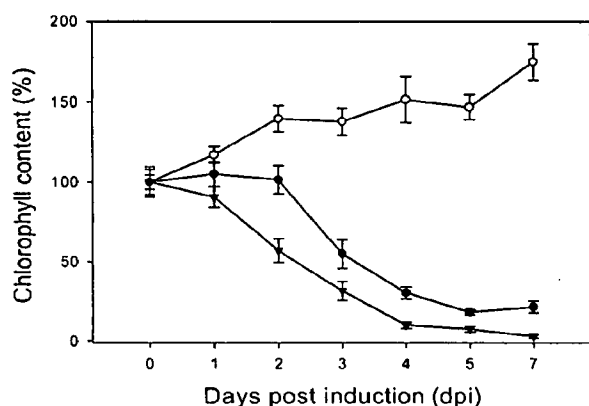


Figure 5. Changes in chl content in *alc-dsRNA* transgenic plants after ethanol induction. Control plants (○), *alc-dsGSA* line 60 (●), and *alc-dsChl I* line 16 (▼) were induced with 100 ml 1% (v/v) ethanol via root drenching, and chl content was measured at the times indicated. Samples were taken from leaves being c. 5 cm in size at the time of induction until 7 dpi. The chl content is expressed as a value relative to that at day 0. Bars show the SD of five replicates.

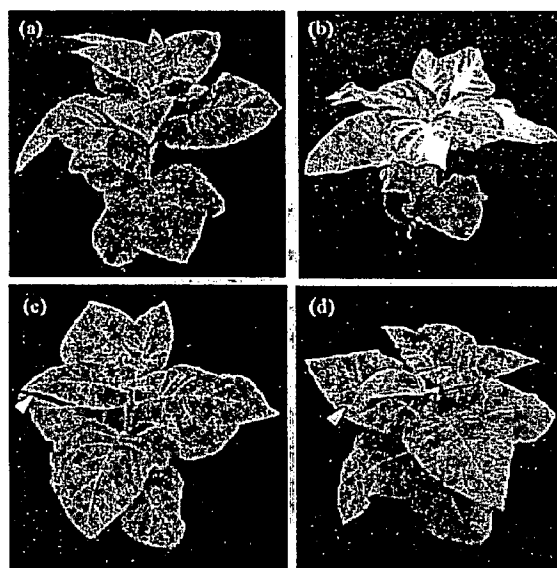


Figure 6. Temporal and spatial control of *Chl I* gene silencing. (a, b) Wild-type (a) and *alc-dsChl I* (b) plants (line 16) were root-drenched with 1% ethanol solution every 2 days. The pictures were taken at 15 dpi. (c, d) Local *Chl I* silencing. The leaf indicated by an arrow was enclosed with 3 ml of 4% (v/v) ethanol for 48 h. (c), wild-type control plant; (d), *alc-dsChl I* plant from line 16.

### Spatial control of gene silencing using the *alc* system

Previous studies demonstrated that spatial induction of *alc*-reporter gene constructs could be achieved by exposing single leaf to ethanol vapor (Sweetman *et al.*, 2002). In order to test whether this was also possible for inducible gene silencing, an individual leaf of an *alc-dsChl I* plant was exposed to ethanol vapor using a similar 'bagging' experiment as described before by Sweetman *et al.*, (2002). As shown in Figure 6(d), the respective leaf displayed the typical symptoms of *Chl I* downregulation observed in whole plant induction experiments. The phenotype was restricted to the treated leaf only, indicating that neither transport of ethanol into adjacent parts of the plant nor spread of silencing occurred. Thus, our data demonstrate that confined vapor treatment allows for spatial control of gene silencing using the *alc* system.

### Discussion

Downregulation of endogenous genes via PTGS using sense or antisense constructs is a crucial tool to assess gene function in transgenic plants. Recent findings indicate that expression of self-complementary hairpin RNAs greatly enhances the efficacy of such experiments (Chuang and Meyerowitz, 2000; Levin *et al.*, 2000; Smith *et al.*, 2000; Stoutjesdijk *et al.*, 2002; Waterhouse *et al.*, 1998). Although

these approaches have been proven to be extremely useful, they are not without problems. One drawback of these studies is that constitutive gene silencing often entails pleiotropic effects on growth and development of the transgenic plants, which complicate the interpretation of the phenotype and might mask true gene function. Furthermore, if expression of the target gene is essential for early growth or regeneration during tissue culture, vital plants might not be recovered. Here, we show that temporal and spatial control of gene silencing in transgenic plants can be achieved by ethanol-inducible expression of dsRNA constructs using the *alc* system.

We used two genes involved in chl biosynthesis, namely *Chl I* and *GSA*, to test downregulation by ethanol-inducible expression of antisense fragments and dsRNA constructs, respectively. Suppression of either of the two target genes was assumed to result in a loss of pigmentation because of reduced chl biosynthesis (Höfgen *et al.*, 1994; Papenbrock *et al.*, 2000). However, only constructs giving rise to dsRNA were effective in triggering gene silencing, as revealed by rapid development of the characteristic phenotype after induction with ethanol. Constructs designed to express conventional antisense fragments failed to interfere with expression of the target gene, which was further confirmed by Northern blotting. This is consistent with the assumption that dsRNA is a much stronger trigger of PTGS than ssRNA, possibly by circumventing the initial conversion of ssRNA into dsRNA by an RNA-dependent RNA polymerase (Béclin *et al.*, 2002; Dalmay *et al.*, 2000). First signs of phenotype development occurred at 36 hpi for *alc-dsChl I* to 48 hpi for *alc-dsGSA*, most likely reflecting differences in mRNA and protein turnover rates of the respective endogene. The time point of phenotype development was highly reproducible in several independent induction experiments (data not shown) and is much more rapid than the 10 days to 3 weeks, which have been reported to be necessary for phenotype induction using various virus-based gene silencing systems (Gosselé *et al.*, 2002; Hiriart *et al.*, 2002; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002). Using a single induction, the phenotype persisted for approximately 9 days, which is considerably shorter than what has been shown for VIGS (Gosselé *et al.*, 2002; Ratcliff *et al.*, 2001; Turnage *et al.*, 2002). However, re-iterated ethanol treatment permits to maintain stable silencing for extended periods, which should enable to follow the full sequence of consequences of reduced gene expression whenever desirable.

One of the most interesting features of gene silencing is that it can act non-cell-autonomously, meaning that it can be induced locally and subsequently spread throughout the organism, implying the existence of a mobile silencing signal (Klahre *et al.*, 2002; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). Little is known about the nature of the signal, but it seems likely that the sequence-specific component is

an RNA (Boutla *et al.*, 2002; Mlotshwa *et al.*, 2002). However, evidence suggests that highly expressed transgenes are much better for systemic silencing than are endogenous genes, suggesting that the amount of target RNA is important in establishing systemic silencing in response to the mobile signal (Palauqui and Vaucheret, 1998; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). This is consistent with the finding that using the *alc* system, local silencing of *Chl I* could be achieved by confined ethanol treatment of a single leaf. In this case, no other parts of the plant but the treated leaf displayed any visible signs of *Chl I* silencing, indicating that no systemic spread of PTGS occurred. Spatial control of gene silencing is desirable in some situations, for instance to study the function of widely expressed genes on an organ or tissue basis without affecting the entire plant. On the other hand, the system allows for the investigation of physiological perturbations on the whole plant level, which were caused by local silencing of a particular gene.

To determine the degree of silencing, which could be achieved by inducible expression of dsRNA constructs on the biochemical level, the decline in chl content in phenotypically affected leaves was taken as an indirect measure for chl biosynthetic capacity. A reduction in chl of approximately 80% was achieved in *alc-dsGSA* plants, which is in the range of what has been reported for transgenic tobacco plants constitutively expressing *GSA* antisense RNA (Höfgen *et al.*, 1994). Transgenic tobacco plants constitutively silenced for *Chl I* were shown to have approximately 40% of the chl content as compared to the control (Papenbrock *et al.*, 2000). Inducible silencing of *Chl I* using the *alc* system reduced the chl content in phenotypically affected leaves to approximately 3% of that before induction, indicating a much stronger effect on *Chl I* expression than that in constitutively silenced plants. It is reasonable to assume that in case of constitutive silencing, there is a considerable selection against the reduction of Mg-chelatase activity below a certain threshold level, which does not support plant growth under autotrophic conditions. Thus, in some cases, inducible PTGS using the *alc* system is likely to provide a valid strategy to obtain a degree of silencing, which otherwise prevents the regeneration of viable transgenics.

Recently, an alternative system to achieve chemical-regulated inducible gene silencing has been described, which takes advantage of 17 $\beta$ -estradiol-inducible recombination to trigger the expression of an intron-containing inverted-repeat RNA (CLX system; Guo *et al.*, 2003). In this case, induction of the system leads to permanent activation of PTGS mimicking expression from a constitutive promoter. However, because of the nature of the inducer, the CLX system might not be readily applicable to soil-grown plants, and thus its use in physiological studies is limited.

In summary, the data presented here demonstrate the utility of the *alc* gene system to achieve transient gene

silencing by inducible expression of dsRNA constructs. The *alc* system offers an enormous flexibility with respect to time point of induction, expression level, spatial control, and duration of expression, and is applicable to a variety of plant species. In contrast to other chemically regulated systems, ethanol, or alternatively acetaldehyde, is comparatively a benign inducer and exerts only minimal physiological side-effects in concentrations necessary for induction (Junker *et al.*, 2003). These attributes greatly enhance the reproducibility of silencing experiments, which is of particular importance in metabolic studies requiring a large population of uniformly silenced individuals. The system provides a powerful tool to investigate molecular and physiological alterations associated with repression of a target gene at temporal and spatial resolution. Thus, dissection of primary and secondary effects of gene silencing should be greatly facilitated, allowing more precise predictions of gene function.

## Experimental procedures

### Transgenic plants, growth, and maintenance

Tobacco plants (*N. tabacum* cv. Samsun NN) were obtained from Vereinigte Saatuchten eG (Ebsdorf, Germany) and grown in tissue culture under a 16-h light/8-h dark regime (irradiance 150  $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ ) at 50% humidity on Murashige and Skoog medium (Sigma, St Louis, MO, USA) containing 2% (w/v) sucrose. Plants in the greenhouse were kept in soil under a light/dark regime of 16 h light (25°C) and 8 h (20°C) dark.

### Plasmid construction and plant transformation

All constructs for plant transformation were cloned into p35S:*alcR*, a derivative of pBin19 (Bevan, 1984), carrying the *alcR* gene from *A. nidulans* between the CaMV 35S promoter and the *nos* terminator (Caddick *et al.*, 1998) using standard procedures (Sambrook *et al.*, 1989). Fragments containing portions of the respective target gene in sense and antisense orientation separated by an intron were initially assembled into a pUC-based vector. To this end, the first intron of the gibberellin 20 (GA20) oxidase gene from *Solanum tuberosum* (kindly provided by S. Biemelt, IPK Gatersleben, Germany) was PCR amplified using the primers 5'-cctgcaggctcgagactagtagatctggtacggacgactactctct-3' and 5'-cc-tgcagggtcgactctagaggatccccatatataatttaagtggaaaa-3'. The oligonucleotides were designed to introduce *Pst*I/*Xho*I/*Spe*I/*Bgl*II sites at the 5' end and *Bam*HI/*Xba*I/*Sac*I/*Pst*I sites at the 3' end, into the resulting PCR product. The 200-bp intron fragment was inserted into a pUC18 vector devoid of the polylinker site via blunt-end ligation resulting in the plasmid pUC-RNAi. A 655-bp fragment of the *N. tabacum Chl I* gene (GenBank Accession number U67064) comprising nucleotides 454–1108 was amplified by PCR from tobacco cDNA and inserted as a *Bam*HI/*Sac*I fragment in sense orientation downstream of the GA20 intron into pUC-RNAi using the before-mentioned restriction sites. The same fragment was inserted in antisense orientation into the *Bgl*II/*Xho*I sites of pUC-RNAi already carrying the *Chl I* sense fragment. Subsequently, the entire fragment comprising sense and antisense fragments of *Chl I* interspersed by the potato GA20 oxidase intron was excised from

pUC-RNAi using the flanking *Pst*I restriction sites and inserted into a pUC-based plasmid between a chimeric *alcA* promoter and a *nos* terminator sequence (Caddick *et al.*, 1998). The resulting *alcA* expression cassette was subsequently inserted into the *Hind*III site of p35S:*alcR* yielding the construct *alc-dsChl I*. An 804-bp fragment of *N. tabacum GSA* (Höfgen *et al.*, 1994; GenBank Accession number X65974) comprising nucleotides 298–1101 of the respective cDNA clone, was amplified by PCR and manipulated as described above to form plasmid *alc-dsGSA*.

To obtain constructs for ethanol-inducible antisense RNA expression, the same fragments as used before were linked in reverse orientation to the chimeric *alcA* promoter, and the entire cassette was subsequently ligated into p35S:*alcR* as above.

Transformation of tobacco plants by *Agrobacterium*-mediated gene transfer using *A. tumefaciens* strain C58C1:pGV2260 was carried out as described previously by Rosahl *et al.*, (1987).

Constructs and biomaterials concerning the ethanol-inducible system are available for academic research purposes subject to satisfactory completion of a material transfer agreement with Syngenta. For further information, contact the Licensing Manager, Syngenta; Jeallots Hill International Research Center, Bracknell, Berkshire RG42 6EY, UK.

### Ethanol induction

Plants (42 days old) cultivated in the greenhouse in 2.5-l pots were induced with 100 ml of 1% (v/v) ethanol solution via root drenching. Normal watering was resumed after application. Samples for RNA, chl, and protein analysis were taken at various time points indicated in the section under Results. If not otherwise stated, young leaves, being approximately 5 cm at the time point of induction, were followed over the time course of the experiment. For spatial induction, an individual leaf was enclosed in a 15 cm  $\times$  10 cm transparent plastic bag with 3 ml of 4% (v/v) ethanol as described previously by Sweetman *et al.*, (2002). The bag was removed after 48 h and phenotype development was monitored by eye.

### RNA analysis

Total RNA was extracted from tobacco leaf material as described by Logemann *et al.*, (1987), and 30  $\mu\text{g}$  per sample was separated on a 1.5% (w/v) formaldehyde-agarose gel using conditions described by Sambrook *et al.*, (1989). After electrophoresis, RNA was transferred to a nitrocellulose membrane (GeneScreen, NEN Life Science Products, Boston, USA) and fixed by UV cross-linking. Filters were pre-hybridized, hybridized, and washed essentially as described by Sweetman *et al.*, (2002). *GSA* and *Chl I* transcripts were detected using a random-primed [ $^{32}\text{P}$ ]-labeled cDNA fragment.

### Protein analysis

Protein extracts were prepared by homogenization of leaf material in a buffer containing 25 mM HEPES, pH 7.0, 12 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 8 mM DTT, 10  $\mu\text{M}$  PMSF, 0.1% Triton, and 10% glycerol. Protein content was determined according to Bradford (1976). After heat denaturation, 30  $\mu\text{g}$  of total protein was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membrane (Poreblot, Macherey und Nagel, Düren, Germany). Immunodetection was carried out using the ECL kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer, using a rabbit

anti-GSA primary antibody (kindly provided by Dr Bernhard Grimm, Humboldt University, Berlin, Germany) and peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA).

#### Chlorophyll determination

Chlorophyll was measured in ethanol extracts and concentrations were determined as described by Lichtenthaler (1987).

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ORIGINAL ARTICLE

Marina Byzova · Christoph Verduyn · Dirk De Brouwer  
Marc De Block

## Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner

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**Abstract** Oilseed rape (*Brassica napus* L.) genotypes with no or small petals are thought to have advantages in photosynthetic activity. The flowers of field-grown oilseed rape form a bright-yellow canopy that reflects and absorbs nearly 60% of the photosynthetically active radiation (PAR), causing a severe yield penalty. Reducing the size of the petals and/or removing the reflecting colour will improve the transmission of PAR to the leaves and is expected to increase the crop productivity. In this study the 'hairpin' RNA-mediated (hpRNA) gene silencing technology was implemented in *Arabidopsis thaliana* (L.) Heynh. and *B. napus* to silence B-type MADS-box floral organ identity genes in a second-whorl-specific manner. In *Arabidopsis*, silencing of B-type MADS-box genes was obtained by expressing *B. napus* *APETALA3* (*BAP3*) or *PISTILLATA* (*BPI*) homologous self-complementary hpRNA constructs under control of the *Arabidopsis* A-type MADS-box gene *APETALA1* (*API*) promoter. In *B. napus*, silencing of the *BPI* gene family was achieved by expressing a similar hpRNA construct as used in *Arabidopsis* under the control of a chimeric promoter consisting of a modified petal-specific *Arabidopsis* *AP3* promoter fragment fused to the *API* promoter. In this way, transgenic plants were generated producing male fertile flowers in which the petals were converted into sepals (*Arabidopsis*) or into sepaloid petals (*B. napus*). These novel flower phenotypes were stable and heritable in both species.

**Keywords** Apetalous · *Arabidopsis* · *Brassica* · Double sepaloid · MADS-box · Petal

**Abbreviations** PAR: photosynthetically active radiation · *ST-LS1*: potato light-inducible tissue-specific ST-LS1 gene · *GUS*:  $\beta$ -glucuronidase

### Introduction

Flowers of oilseed rape (*Brassica napus*) have four well-developed bright-yellow petals. During flowering time, flowers form a very bright-yellow layer that reflects and absorbs solar radiation. As consequence, only 24% of the photosynthetically active radiation (PAR) reaches the leaf canopy (Chapman et al. 1984). This accelerates leaf and bract senescence, reduces dry matter accumulation, and lowers seed set (Daniels et al. 1986).

A few strategies to improve the photosynthetic efficiency of oilseed rape by utilising different apetalous variants (Buzza 1983; Jiang and Becker 2003) or the *stamenoid petal* (stap) variant with flowers bearing staminoid petals (Fray et al. 1997) have been proposed. Physiological analyses have revealed the potential benefit of such a petalless flower phenotype on *B. napus* yield (Rao et al. 1991; Fray et al. 1995).

The currently used apetalous genotypes are controlled either by two recessive genes (Fray et al. 1996) or by an interaction of cytoplasmic genes and two pairs of nuclear genes (Jiang and Becker 2003). This genetic complexity makes it difficult to fully implement the apetalous trait into commercial rapeseed varieties. Additionally, the apetalous character appears to be unstable under field conditions at high temperatures and in long days (Rao et al. 1991). The *B. napus* *stap* variant also possesses poor agronomic attributes, such as deformed leaves and poor vigour (Fray et al. 1997).

A more promising strategy to improve PAR transmission in oilseed rape would be the use of a single dominant gene that converts the bright-yellow petals into small non-light reflecting structures such as sepals. Such an organ conversion is preferable over the removal of the petals to avoid interfering with insect pollination. Pierre et al. (1996) have shown that honeybees, the main

M. Byzova · C. Verduyn · D. De Brouwer  
M. De Block (✉)  
Bayer BioScience N.V., Jozef Plateastraat 22,  
9000 Gent, Belgium  
E-mail: marc.deblock@bayercropscience.com  
Fax: + 32-9-2240694

pollinators on oilseed rape, do not crawl over the anthers and stigma of apetalous flowers as they do in petalous ones but often insert their tongues between the sepals to collect the nectar. In this way pollination might be reduced, resulting in a lower seed set.

The molecular mechanisms governing floral organ identity are well understood. According to the "A-E" model, the organ identity of each floral whorl is determined by a unique combination of four organ identity activities, called A, B, C and E (Weigel and Meyerowitz 1994; Pelaz et al. 2000; Jack 2001; Theissen 2001; Fig. 1). Expression of the (A)-type genes specifies sepal formation. The combination of (A + B + E) activities spec-

ifies the formation of petals, while combined (B + C + E) functions specify stamen formation. Expression of the (C + E)-type genes determines the development of carpels (Fig. 1). All types of organ identity genes have been cloned from *Arabidopsis*. An example of the A-type gene is *AP1* (Mandel et al. 1992). The B-type genes are *AP3* (Jack et al. 1992) and *PI* (Goto and Meyerowitz 1994), and the C-type gene is *AGAMOUS* (*AG*) (Yanofsky et al. 1990). The E-function is provided by three *SEPALLATA* genes (Pelaz et al. 2000). All these genes are transcription factors belonging to the MADS-box gene family.

In this paper, silencing of the B-type MADS-box genes in a second-whorl-specific manner was obtained in both *Arabidopsis* and *B. napus* flowers by expressing a *B. napus* B-type gene hpRNA construct under control of an *Arabidopsis* A-type MADS-box gene promoter (Fig. 1). In this way, *Arabidopsis* lines with double sepaloid flowers and *B. napus* lines with flowers in which petals are converted into sepaloid petals were generated. The novel flower phenotypes were stable and heritable in both species.

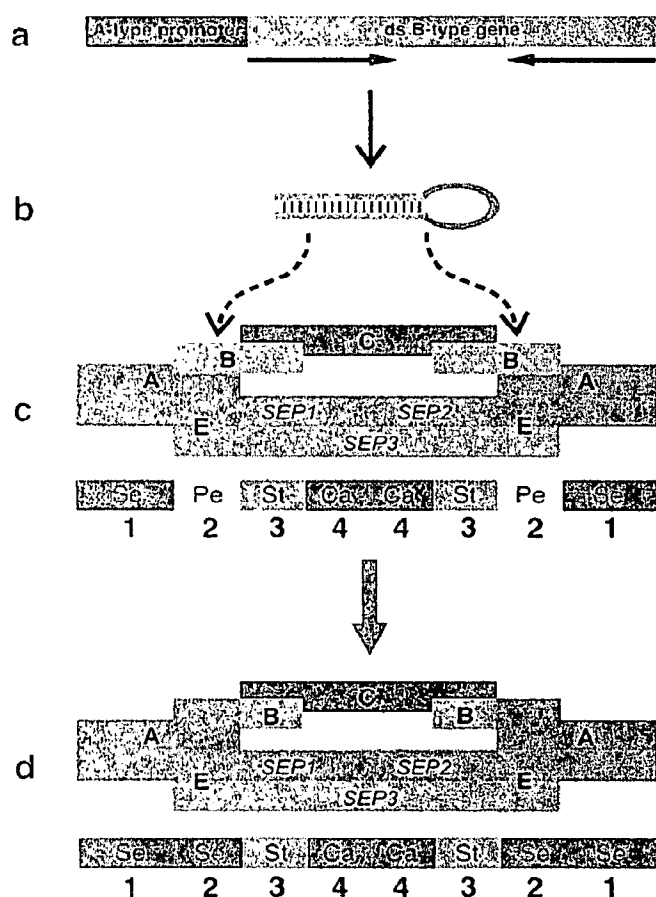


Fig. 1a-d Experimental strategy. a Representation of the basic construct used in this study. A DNA fragment of a 3'-coding region of a B-type MADS-box gene (yellow box) was sub-cloned as an inverted repeat (directions are indicated by arrows) with a part of the GUS gene or the intron IV2 from gene ST-LS1 (Vancanneyt et al. 1990) as a spacer (blue box). The constructs were driven by an A-type MADS-box gene promoter (green box). b Transcripts produced by the construct are predicted to form a hairpin structure. c Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in wild-type plants. d Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in transgenic plants. In transgenic plants, down-regulation of the B-type function in the second whorl only leads to development of sepals instead of petals. Numbers indicate whorls. Se sepals, Pe petals, St stamens, Ca carpels

## Materials and methods

### Plant material

*Arabidopsis thaliana* (L.) Heynh. ecotype C24, kindly provided by Dr. M. Van Lijsebettens (VIB, Gent, Belgium), and the double haploid *Brassica napus* L. line cv. Simon (Bayer BioScience N.V., Gent, Belgium) were used in this study.

### Plasmid construction

The 3'-coding regions of the *BAP3* and *BPI* genes were cloned by means of RT-PCR performed on total RNA isolated from *B. napus* flower buds. RT-PCR was performed according to the protocol of the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). *AP3* cDNA-specific primers:

- 5'-CGCACTCAGATTAAGCAGAGGC-3' and
- 5'-GGAAGGTAATGATGTCAGAGGC-3'

and *PI* cDNA-specific primers:

- 5'-GGGAGAAGATATACAGTCTCTCAAC-3' and
- 5'-GAATCGGTTGCACTCTATATCC-3'

were chosen based on the published sequences (Jack et al. 1992, GenBank Accession D30807; Goto and Meyerowitz 1994, GenBank Accession M86337). In the pAP1::hpBAP3 construct, one of the *BAP3*-specific DNA fragments, 380-bp in length, was cloned as an inverted repeat with the  $\beta$ -glucuronidase (GUS) fragment containing nucleotides 744-975 as a spacer. In the pAP1::hpBPI construct, one of the *BPI*-specific fragments, 255-bp in length, was cloned as an inverted repeat with the intron IV2 from the potato light-inducible tissue-specific gene ST-LS1, 251-bp in length, as a spacer (Vancanneyt et al. 1990). In the pAP1::hpBAP3 and pAP1::hpBPI constructs, gene-specific structures were driven by a 1,182-bp fragment of the *AP1* promoter. The fragment of the *AP1* promoter (-1182 to +1) was cloned by means of PCR from pKY65 plasmid kindly provided by Martin Yanofsky. In pAP3-AP1::hpBPI fragments of the *AP3* promoter, containing nucleotides -727 to -556 and -224 to -1 were cloned by PCR based on

the published sequence (Irish and Yamamoto 1995, GenBank Accession U30729) and linked to the 5'-end of the *API* promoter. Plasmid constructs were introduced into *Agrobacterium tumefaciens* strain C58C1rif by electroporation.

#### Plant transformation

The transformations of *A. thaliana* and *B. napus* were essentially done as described by Valvekens et al. (1992) and De Block et al. (1989), respectively.

#### Cytology

The embedding was done in Historesin as advised by the manufacturer (Leica, Heidelberg, Germany). Sections 5  $\mu$ m thick were stained with 0.05% toluidine blue.

#### In situ hybridization

Embedding in methacrylate, sectioning, and the removal of the plastic were essentially done as described by Baskin et al. (1992). The in situ hybridizations on 7- $\mu$ m sections were essentially done as described by De Block and De Brouwer (1993).

#### Microscopy

Sections were examined with an Axioplan (Zeiss, Jena, Germany) microscope equipped with Normaski differential interference contrast.

#### Spectrophotometric determination of chlorophyll

The total chlorophyll (*a* + *b*) content was measured as described by Bruisma (1963).

## Results

### General strategy: silencing the B-type MADS-box genes in a second floral whorl-specific manner

To convert petals into sepals without interfering with anther development, the strategy outlined in Fig. 1 was used. Following the A–E flower development model it is expected that silencing of a B-type MADS-box gene, *AP3* or *PI*, in the second whorl will redirect the development of petals into sepals. This could be obtained by expressing in the second, but not in the third whorl self-complementary 'hairpin' RNA (hpRNA) constructs containing *AP3*- and/or *PI*-specific sequences. Down-regulation of the B-type MADS-box genes in the third whorl has to be avoided to maintain normal male fertility. For this purpose an A-type promoter driving the expression of the hpRNA construct could be used.

Starting from the *PI* and *AP3* sequences (Jack et al. 1992; Goto and Meyerowitz 1994), we identified in the amphidiploid *B. napus* five *AP3*-like (*BAP3*) and three *PI*-like (*BPI*) genes that were actively expressed during flower development (data not shown). Fragments of the 3'-coding region of the *BAP3* and *BPI* genes were isolated. The nucleotide sequence similarity between

members of the same B-type MADS-box gene subfamily turned out to be on average 95%. Each *B. napus* gene subfamily shared with its unique *Arabidopsis* counterpart about 91% sequence similarity, containing multiple blocks of more than 20 bases of perfect homology. This high sequence similarity should be sufficient to silence the target genes in both *Arabidopsis* and *B. napus* by using the same hpRNA constructs (Helliwell and Waterhouse 2003). The feasibility of the strategy to convert petals into sepals by silencing the B-type MADS-box genes only in the second floral whorl was first evaluated in the model plant *Arabidopsis thaliana*.

### Generation of *Arabidopsis* transgenic lines with male fertile double sepaloïd flowers

To make constructs that produce hpRNA B-type MADS-box gene transcript, the 3'-coding regions of one *BAP3* and one *BPI* gene, were subcloned as an inverted repeat (see Materials and methods). Both hpBAP3 and hpBPI gene-specific sequences were driven by a 1.1-kb promoter fragment of the *Arabidopsis API* gene. The resulting pAPI::hpBAP3 and pAPI::hpBPI constructs were introduced separately into *Arabidopsis*.

A total of 125 pAPI::hpBAP3 and 56 pAPI::hpBPI transgenic lines was generated. All the plants were normal in terms of vegetative growth while they had morphological changes in flower organs. 16.9% of the pAPI::hpBPI and 5.6% of the pAPI::hpBAP3 lines exhibited the desirable double sepaloïd phenotype (Fig. 2b). Instead of petals, sepals developed in the second floral whorl, indistinguishable from those of the first whorl except for their slightly smaller size. Despite their transformation, these organs developed in the positions and on a time course characteristics of petals. Some other pAPI::hpBAP3 *T*<sub>0</sub> plants had a range of phenotypes related to the severity of homeotic transformations observed in petal and stamen development. 10.4% of the pAPI::hpBAP3 lines produced flowers with short white petals and 20% of the lines had homeotic aberrations in stamens ranging from weak carpelloïd to complete transformation of stamens into carpels (Table 1). In contrast to the pAPI::hpBAP3 lines, no aberrations in the third floral whorl were observed in the pAPI::hpBPI transgenic plants (Table 1).

Microscopic analysis of cross-sections of mature pAPI::hpBPI double sepaloïd flowers revealed that the mesophyll cells of the second-whorl organs were sepaloïd in nature, as indicated by the presence of chloroplasts and their larger size than those normally found in wild-type petals. The abaxial epidermis was like that of sepals, consisting of stomata and irregularly shaped cells (Fig. 2d). The same results were obtained for pAPI::hpBAP3 double sepaloïd flowers (data not shown).

To confirm that the double sepaloïd phenotype of *Arabidopsis* transgenic plants was caused by depletion of expression of endogenous B-type homeotic genes in the



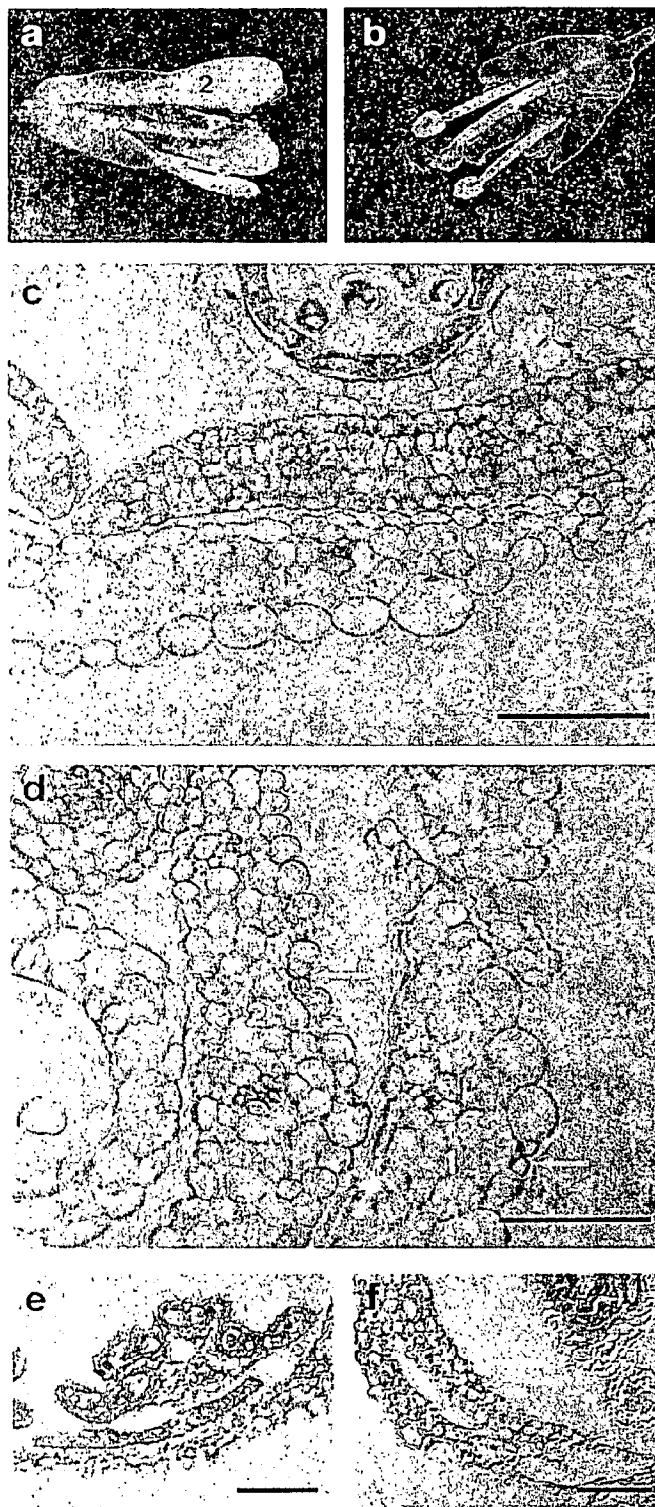


Fig. 2a–f Analysis of the double sepaloid pAPI::hpBPI *Arabidopsis thaliana* flowers. a C24 *Arabidopsis* wild-type mature flower. b Mature transgenic flower. Second-whorl organs are sepals (arrow) that are slightly smaller than the true sepals. c, d Cytological transverse sections taken approximately in the middle of anthers of flower buds at stage 12. c Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of the second-whorl petals are smaller than those of sepals developed in the first whorl. Abaxial epidermal cells of petals are regular in shape. d Cellular morphology of first- and second-whorl organs of a transgenic flower. Mesophyll and epidermal cells of the second-whorl organs are slightly smaller in size than cells of the first-whorl sepals. The shape of the cells of the second-whorl sepals is similar to those of the first-whorl sepals. Stomata (arrows) are present in the abaxial epidermis of the second-whorl organs as in normal first-whorl sepals. e, f In situ analysis of *PI* expression in transverse sections of wild-type and transgenic flowers. The hybridisation signal is confined to the second- and the third-whorl organs in wild-type flowers (e). In transgenic flowers (f) *PI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 50 µm (c, d, f), 100 µm (e)

from stage 3 (Smyth et al. 1990) in second- third- and fourth-whorl primordia. In the second and third whorls it persists until anthesis (Goto and Meyerowitz 1994). In pAPI::hpBPI double sepaloid flowers the *PI* transcript was not detectable in the second-whorl primordia at any of the stages examined (Fig. 2f). Expression of *PI* in developing stamens was similar to that observed in the wild type.

As it has been shown that expression of both *PI* and *AP3* genes is reduced when either the *PI* or *AP3* gene is mutated (Jack et al. 1992; Goto and Meyerowitz 1994), we anticipated that inhibition of expression of one of the B-type MADS-box genes in a tissue where both genes are active would lead to reduction of expression of the counterpart gene in the same manner. To verify this hypothesis, in situ hybridization of the pAPI::hpBPI flowers using the *BAP3*-specific fragment as a probe was performed. As was predicted, *AP3* RNA was not detected in the second whorl of developing organs. However, no reduction in the level of the *AP3* mRNA was observed in stamens (data not shown).

Heritability and stability of the double sepaloid trait was tested by self-pollination. The trait was heritable and in the case of pAPI::hpBPI stable through the T<sub>1</sub> and T<sub>2</sub> generations. In the case of pAPI::hpBAP3 some T<sub>1</sub> and T<sub>2</sub> lines produced flowers with homeotic aberrations in stamens, as previously observed in the T<sub>0</sub> plants.

**In *B. napus*, silencing of B-type MADS-box genes in the second whorl results in the transformation of petals into sepaloid petals**

To evaluate whether the expression of the pAPI::hpBAP3 and pAPI::hpBPI genes would also result in a double sepaloid phenotype in *B. napus*, 48 and 53 transgenic lines, respectively, were generated.

All the pAPI::hpBAP3 lines had wild-type flowers. Among the pAPI::hpBPI transgenic lines, 22.6%

second whorl, the *PI* mRNA expression pattern in pAPI::hpBPI was examined by in situ hybridization. In wild-type *Arabidopsis* flowers, *PI* mRNA is detected

**Table 1** Phenotypic analysis of *T<sub>0</sub>* *Arabidopsis thaliana* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)		
		Double sepaloid Fertile	Double sepaloid Partially male sterile <sup>a</sup>	Short petals Fertile
pAPI::hpBPI	56	16.9	< 2	18.9
pAPI::hpBAP3	125	5.6	20	10.4

<sup>a</sup>Range of aberrations in stamens from mild to complete conversion of stamens into carpels

exhibited an apetalous or partially apetalous phenotype characterised by the appearance of flowers without petals or bearing 1, 2 or 3 petals only (Table 2). Frequently, the petals were significantly smaller and narrower than those from wild type (data not shown). However, this phenotype was unstable and not heritable.

The absence of the double sepaloid phenotype in transgenic *B. napus* lines with the same constructs used in *Arabidopsis* could be due to an inability of the *Arabidopsis* *API* promoter to direct transcription of adequate amounts of double-stranded transcripts necessary to trigger silencing of all target *BAP3* or *BPI* genes expressed in rapeseed flowers. Starting from this hypothesis, a new construct was generated that could produce higher amounts of hpRNA. Because the pAPI::hpBAP3 *B. napus* transgenic plants did not exhibit any phenotypes different from those of wild-type plants, and in *Arabidopsis* the most stable double sepaloid flower phenotype was obtained with the hpBPI construct, we continued only with the hpRNA *BPI* gene.

To enhance the level of expression of hpBPI specifically in the second whorl, an *Arabidopsis* modified *AP3* regulatory fragment was added to the *API* promoter.

Discrete *cis*-acting elements regulating spatial and temporal expression of the *Arabidopsis* *AP3* gene have been identified (Hill et al. 1998; Tilly et al. 1998). Based on these data the positive regulator of the *AP3* expression during the early stages of flower development was combined with the petal-specific regulatory region (see Materials and methods). The modified *AP3* promoter was introduced in the pAPI::hpBPI construct directly upstream of the *API* sequence. This pΔAP3-AP1::hpBPI construct was transformed into *B. napus*.

Of the 125 primary transformants, 11.2% produced flowers with aberrant second-whorl organs. Of these 11.2% lines, half (5.6%) produced flowers in which petals were converted into sepaloid petals (Fig. 3a, Table 2). These organs were yellowish-green, indicating the presence of chloroplasts in their cells that is characteristic of wild-type sepals. The size of the sepaloid petals was comparable to the size of true sepals. These organs

were narrow and almost strap-like in shape, like sepals, but had a small lamina and base, characteristic of a petal. In addition the lamina portion was wrinkled (Fig. 3a).

The aberrant *B. napus* flowers with sepaloid petals were analysed microscopically to verify the identity of tissues in the second-whorl organs. As shown in Fig. 3c the size and the shape of epidermal and mesophyll cells of these organs were indistinguishable from the first-whorl sepals. Moreover, the mesophyll cells of the sepaloid petals contained a large number of chloroplasts (Fig. 3d).

In addition, spectrophotometric analysis of chlorophyll fluorescence, which was done on the first and the second floral organs of transgenic plants, revealed that chlorophyll content in the sepaloid petals is only 30% less than in the true wild-type sepals (data not shown).

In situ hybridization of flower sections with a *BPI*-specific probe confirmed the absence of a detectable level of *BPI* gene expression in the second whorl of the transgenic flowers, indicating that the complete *BPI* gene family was down-regulated (Fig. 3e).

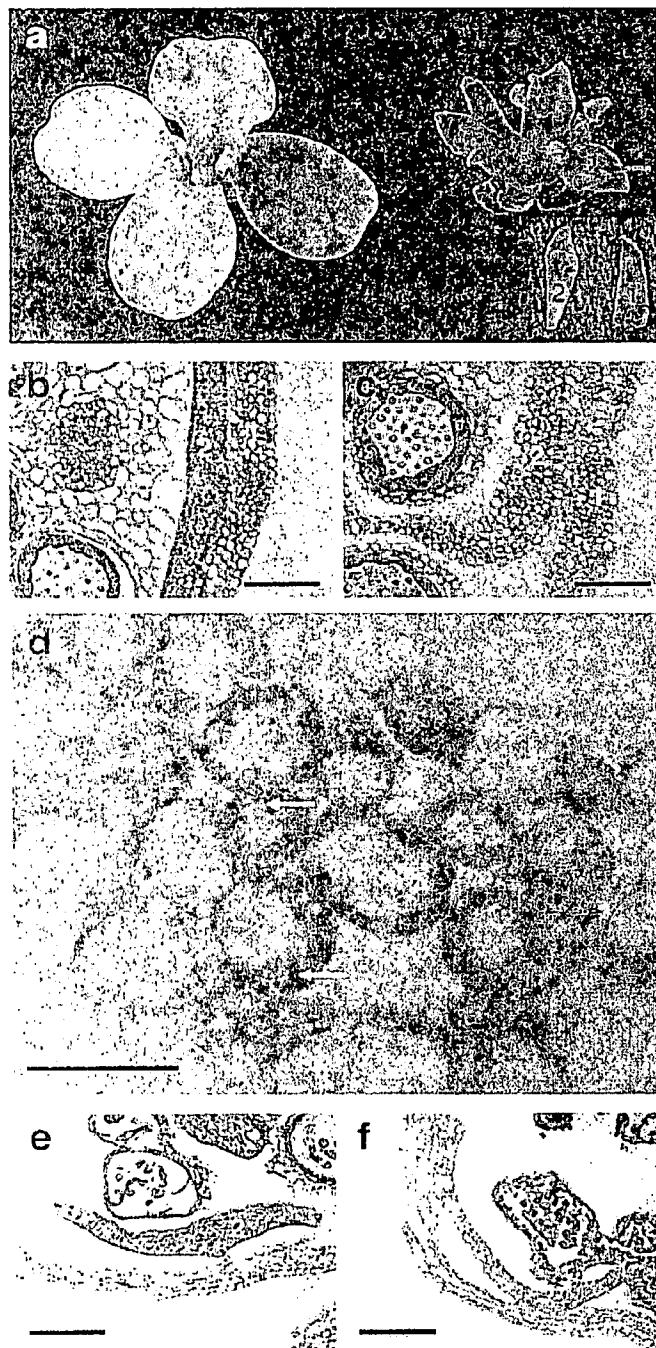
The other half of the 11.2% transgenic pΔAP3-AP1::hpBPI lines exhibited partial apetalous and apetalous phenotypes similar to those observed in pAPI::hpBPI transgenic plants (Table 2).

The flower phenotype with sepaloid petals is a stable trait in *B. napus* transgenic plants

The stability of transformation of petals to sepaloid petals in *B. napus* was tested for six lines, of which the original *T<sub>0</sub>* plants had flowers with sepaloid petals and contained only one copy the pΔAP3-AP1::hpBPI transgene. The *T<sub>0</sub>* plants were first maintained by selfing. The transgenic plants of these *T<sub>1</sub>* generations had flowers with sepaloid petals while the azygous segregants had normal wild-type flowers. For each line ten transgenic plants of the *T<sub>1</sub>* generation were backcrossed with the original non-transgenic double haploid *B. napus* line cv.

**Table 2** Phenotypic analysis of *T<sub>0</sub>* *Brassica napus* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)	
		Sepaloid petals	Apetalous/partially apetalous
pAPI::hpBAP3	48	0	0
pAPI::hpBPI	53	0	22.6
pΔAP3-AP1::hpBPI	125	5.6	5.6



Simon. Depending on whether the  $T_1$  plant used was homo- or heterozygous for the transgene, all or 50% of the  $F_1$  plants, respectively, had sepaloid petals in their flowers. A second backcross was done with 15 plants of each line. As expected, in the  $F_2$  generations there was a 1:1 segregation of wild-type plants and plants with sepaloid petals. The flower phenotype of the transgenic  $F_2$  plants was identical to those of the  $T_0$ ,  $T_1$  and  $F_1$  transgenic plants.

**Fig. 3a–e** Analysis of the pAP3-AP1::hpBPI *B. napus* flowers. **a** Morphological features of *Brassica napus* flowers: mature wild-type flower (left), mature flower of a transgenic plant (right). The second-whorl organs of a transgenic flower are yellowish-green sepaloid petals (arrow). The size of these organs is similar to sepals developed in the first whorl, but the lamina-base structure can still be distinguished (for comparison see the detached organs in the bottom right corner: the second-whorl organ (left), the first-whorl organ (right) of a transgenic flower). **b–d** Cytological transverse sections taken approximately in the middle of anthers at the early yellow bud stage (Smith and Scarisbrick 1990). **b** Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of petals are smaller than those of sepals. Epidermal cells of petals are regular in shape. **c** Cellular morphology of the first- and second-whorl organs of a transgenic plant. The shape and the size of mesophyll and epidermal cells of the second-whorl organs are similar to those of the first-whorl sepals. **d** Cytology of a sepaloid petal showing the presence of chloroplasts (two examples indicated by arrows) in the mesophyll cells. **e, f** In situ analysis of BPI expression on transverse sections of wild-type and transgenic flowers. The hybridization signal is confined to the second- and the third-whorl organs in wild-type flowers (**e**). In transgenic flowers (**f**) BPI expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 20  $\mu$ m (**d**), 100  $\mu$ m (**b, c**), 200  $\mu$ m (**e, f**)

## Discussion

The hpRNA-mediated gene silencing technology has been proven to be a very efficient tool for gene discovery and functional genomics in diverse organisms such as fungi (Pickford et al. 2002), nematodes (Bargmann 2001), and animals (Harborth et al. 2001). In plants this technology has been used successfully to generate virus resistance (Waterhouse et al. 1998) as well as to obtain consistent and profound inhibition of the expression of transgenes and endogenous genes (Levin et al. 2000; Smith et al. 2000; Wesley et al. 2001; Liu et al. 2002).

Chuang and Meyerowitz (2000) demonstrated that the hpRNA-mediated silencing technology could be used to interfere with flower development. A range of aberrant flower phenotypes was obtained by down-regulating the floral organ genes *AGAMOUS*, *CLAVATA3*, *APET-ALAI*, and *PERIANTHIA* using hpRNA constructs driven by the constitutive 35S and nopaline synthase promoters. Recently, it has been shown that the hpRNA-mediated silencing technique can be used to silence genes in an organ-specific way. The fatty acid composition of *Arabidopsis* and cotton seeds was modified by down-regulating the seed expression of two fatty acid desaturase genes using hpRNA constructs driven by seed-specific promoters (Liu et al. 2002; Stoutjesdijk et al. 2002).

In this article we present for the first time to our knowledge implementation of the hpRNA-mediated technology to silence a multigene family in a floral whorl-specific manner. Silencing of the B-type MADS-box genes that are present in single copy in *Arabidopsis* but are present in multiple copies in *B. napus* causes complete transformation of petals to sepals in *Arabidopsis* and partial transformation in *B. napus*. This flower phenotype is stable and heritable in both species.

In *Arabidopsis*, unlike the silencing of the *PI* gene, silencing of the *AP3* gene results in homeotic aberrations in anthers in 20% of the cases. This implies that in these lines partial silencing of the *AP3* gene also occurs in developing stamens. These results can be attributed to two possibilities. First, in wild-type *Arabidopsis* flowers *API* is expressed during early floral stages throughout all four whorls and is down-regulated in whorls 3 and 4 by the *AG* gene during stage 3, persisting in whorls 1 and 2 only (Mandel et al. 1992; Bowman et al. 1993). However, in contrast to the endogenous promoter, the smaller *API* promoter fragment we used might have some activity in the central whorls after stage 3 as proposed by Yun et al. (2002). The activity of the pAPI::hpBAP3 gene might have led to down-regulation of *AP3* in the third floral whorl. Alternatively, an aberrant stamen development in the pAPI::hpBAP3 transgenic plants might be the result of the spreading of a silencing signal between floral whorls.

Both hypotheses imply that a certain amount of dsRNA of the *AP3* gene present in the third whorl of transgenic flowers is sufficient to trigger silencing of *AP3*. This is not the case for the *PI* gene, for which the down-regulation did not result in aberrant anther phenotype. *PI* and *AP3* are both expressed in developing petals and stamens. However *PI* expression levels are similar in both whorls, whereas *AP3* expression is lower in developing stamens than in petals (Zhou et al. 2002). It may be that for this reason a lower threshold concentration of hpRNA is required in stamens to provoke a partial inhibition of the *AP3* gene expression.

Although systemic spreading of silencing may be a concern for implementation of the hpRNA-mediated silencing technology in tissue-specific applications in plants (Wang and Waterhouse 2002), the stability of the aberrant flower phenotype throughout development of our transgenic plants indicates that at least in the case of the B-type MADS-box genes there is no significant spreading of silencing between the meristems of adjacent floral organs.

Another phenomenon that might limit application of the hpRNA gene silencing technique is spreading of RNA targeting. During this process spreading of the RNA silencing signal occurs from the initial target sequence into the adjacent 5' and 3' regions (Jones et al. 1999; Vaistij et al. 2002). This may result in the participation of the entire transcribed region of the target gene in the RNA silencing process. As a consequence, expression of other homologous genes can be inhibited. Based on this hypothesis and the fact that different types of MADS-box genes share a high percentage of homology at the MADS-box regions (Purugganan et al. 1995), target-site spreading along the *AP3* or *PI* transcribed sequences would lead to silencing of not only *AP3* and *PI* but also of other MADS-box genes that are expressed in the developing second-whorl organs. In this case petals will be converted not only into sepals but also into organs with staminoid and/or carpeloid and/or other aberrant

structures. The absence of such phenotypes in our transgenic plants suggests that silencing of B-type MADS-box genes was not associated with the spreading of RNA targeting. The absence of the target-site spreading process was also observed by Vaistij et al. (2002) for the ribulose-1,5-bisphosphate carboxylase/oxygenase and phytoene desaturase genes. These results demonstrate that the hpRNA-mediated gene silencing technology can be applied not only to silence all genes of a multigene family but also to silence specifically a single member of a subfamily or even of a multigene family.

*B. napus* plants transformed with the improved pΔAP3-API::hpBPI construct have small yellowish-green sepaloid petals in the second whorl. Although mesophyll and epidermal cells of these sepaloid petals are sepaloid in morphology, the light-yellow colour suggests that some petal-specific biochemical pathways are still active in the cells of these organs. In addition, the small lamina and base of these organs are petal characteristics. It might be that undetectable levels of *BPI* transcripts are still sufficient for maintenance of some petaloid features.

Recently, in *Arabidopsis* an alternative approach was used to interfere with the expression of *AP3* in a second-whorl-specific manner (Guan et al. 2002). A zinc finger protein designed to bind to a region upstream of *AP3* was fused to the human transcriptional repression domain of mSIN3. When the *API* promoter was used to drive the expression of this artificial zinc finger transcription factor, flowers were obtained that were partially apetalous or that contained some sepaloid petals. Although the use of synthetic transcription factors is a promising approach to interfere with gene regulation, high expression levels of these transcription factors are probably needed to obtain a full phenotype by gene repression. Due to technical limitations the use of such artificial transcription factors is less feasible when multiple genes with redundant function, like the B-type MADS-box genes in *B. napus*, have to be repressed.

Theoretically, in *Arabidopsis* a double sepaloid flower phenotype may also be obtained by silencing the *SEP-ALLATA* genes in the second whorl (Fig. 1). However, due to the redundant function of the *SEPALLATA* genes, all three genes would have to be silenced together (Pelaz et al. 2000).

In conclusion, *Arabidopsis* and *B. napus* lines with a flower phenotype that is, respectively, double sepaloid or has sepaloid petals, and that is male fertile and stable in subsequent generations can be obtained by a hpRNA-mediated gene silencing of the *PISTILLATA* gene exclusively in the second floral whorl. Further physiological studies of *B. napus* transgenic lines will allow quantification of the effect of the flower architecture with sepaloid petals on the distribution of PAR and on other important agronomic features such as pollination and overall seed yield.

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# Making a better RNAi vector for *Drosophila*: use of intron spacers

Young Sik Lee and Richard W. Carthew\*

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2153 North Campus Drive, Evanston, Illinois 60208, USA

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## Abstract

Double-stranded RNA induces sequence-specific inhibition of gene expression at a posttranscriptional level in eukaryotes (RNAi). This natural phenomenon has been developed into a tool for studying gene function in several model organisms, including *Drosophila melanogaster*. Transgenes bearing inverted repeats are able to exert an RNAi effect in *Drosophila*, but cloning difficulties and inconsistent silencing complicate the method. We have constructed a transgene containing inverted repeats separated by a functional intron such that mRNA produced by the transgene is predicted to form loopless hairpin RNA following splicing. A single copy of the transgene effectively and uniformly silences expression of a target gene (*white*) in transgenic flies. We have developed a vector that is designed to produce intron-spliced hairpin RNA corresponding to any *Drosophila* gene. The vector is under control of the upstream activating sequence (UAS) of the yeast transcriptional activator GAL4. The UAS/GAL4 system allows hairpin RNA to conditionally silence gene expression in *Drosophila* in a tissue-specific manner. Moreover, the presence of the intron spacer greatly enhances the stability of inverted-repeat sequences in bacteria, facilitating the cloning procedure.  
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## 1. Introduction

The sequencing of the *Drosophila melanogaster* genome provides an exceptional opportunity to analyze the different functions governed by its genes [1]. Traditionally, genes are characterized by loss-of-function phenotypes caused by mutations that are induced randomly by chemical, physical, or insertional mutagenesis. The annotated sequence of the *Drosophila* genome enables reverse-genetic approaches to be used on a genome-wide scale to generate loss-of-function phenotypes. Targeted gene knockouts have recently been described in *Drosophila* [2,3], but this approach is laborious and does not allow for conditional silencing of gene expression.

Recently, RNA interference (RNAi) has been demonstrated to be an effective reverse-genetic approach to generating loss-of-function phenotypes. The presence of double-stranded RNA (dsRNA) causes the sequence-specific posttranscriptional silencing of a corresponding gene in a variety of organisms [4]. Thus, RNAi is used to

inactivate genes of interest and provides a powerful tool to study gene function. Injection of dsRNA into *Drosophila* embryos silences gene activity effectively, but its effect is transient and is not inherited in the next generation [5,6]. To overcome this problem, methods have been developed to express dsRNA stably in transgenic *Drosophila*. Most of these methods employ transgenes having an inverted-repeat (IR) configuration, which are able to produce dsRNA as extended hairpin RNA [7–10]. An alternative method has used a transgene that is symmetrically transcribed from opposing promoters [11]. A general problem with these methods is that transgenic lines often induce a variable RNAi silencing effect that exhibits incomplete penetrance and expressivity. Consequently, the copy number of silencing transgenes usually needs to be increased to observe uniform and complete gene silencing. Moreover, it is often difficult to make stable recombinant plasmids containing IRs in *Escherichia coli*. Introduction of a spacer sequence between the repeats helps stabilize some recombinant plasmids, but there are still significant reported stability problems.

In this paper, we describe an IR-based transgene designed such that the repeats are separated by a functional intron and thus are defined exons. We report that,

\* Corresponding author. Fax: 1-847-467-1380.

E-mail address: [r-carthew@northwestern.edu](mailto:r-carthew@northwestern.edu) (R.W. Carthew).

in *Drosophila*, the transgene is a powerful repressor of gene activity in vivo, with one copy of the transgene exerting uniformly strong silencing. We further describe a modular system combining GAL4-regulated gene expression with conditional production of the silencing dsRNA to allow systematic RNAi in *Drosophila* using IR exons. With this system, inhibition of gene activity is flexibly induced in any spatial or temporal pattern, allowing for precise disruption of gene function. This technique could potentially be a powerful and economical approach to studying gene function in *Drosophila* and to manipulating gene function in specific tissues of postembryonic individuals.

## 2. First-generation transgenic RNAi in *Drosophila*

We initially developed a method to express dsRNA as extended hairpin-loop RNA [10]. Its design was modeled on the successful application of hairpin RNAs in generating RNAi in plants and the nematode *Caenorhabditis elegans* by expression of transgenes with IR sequences. To facilitate cloning of IRs into recombinant plasmids, we placed a 5-base nonpalindromic sequence centered at the axis of dyad symmetry that was a *Sfi*I site, GGCCATCTAGGCC (Fig. 1). This allowed us to easily ligate gene fragments together in inverted orientation, and it increased the stability of the IR DNA during plasmid replication. Sequence repeats are often deleted in *E. coli* because cruciform intermediates form during replication of plasmid DNA and are excised by the *sbcBC* gene products. Insertion of nonrepetitive se-

quence greater than 4 bp in length between IRs inhibits cruciform excision during replication [12]. Recombinant plasmids were replicated in a recombination-deficient strain. No strain is guaranteed to propagate all recombinant clones, but the SURE strain (Stratagene) is deficient in *recBC sbcBC* and eliminates all known restriction systems. Other strains we used were JM103 and JM105, which are also mutant for *sbcBC*.

To construct an IR transgene, the IR fragment is first cloned into a generic high-copy plasmid vector such as pBluescript (Stratagene) by directional *Eco*RI–*Xho*I two-way ligation. Stable recombinants are selected, and then the IR fragment is shuttled from pBluescript into the *Drosophila* transformation plasmid vector pUAST [13]. We found it more difficult to directly clone IR fragments made in vitro into pUAST, which we circumvented by shuttling the fragment first through pBluescript. On the 5' side of the multicloning site, pUAST contains a *Drosophila* promoter linked to GAL4-responsive upstream activating sequence (UAS) enhancer repeats and on the 3' side of the multicloning site, pUAST contains a polyadenylation signal sequence. Recombinant plasmids are then injected with helper plasmid into *Drosophila* embryos and transformant flies are generated by standard P element transformation [14]. Cloning the IR into a UAS vector allowed us to use the modular design of the GAL4/UAS system in *Drosophila* for misexpressing transgenes. Many useful lines of *Drosophila* express the yeast GAL4 protein in a variety of cells/tissues at various stages of the fly life cycle [13]. GAL4 acts as a sequence-specific transcription activator in *Drosophila*. The GAL4 line is

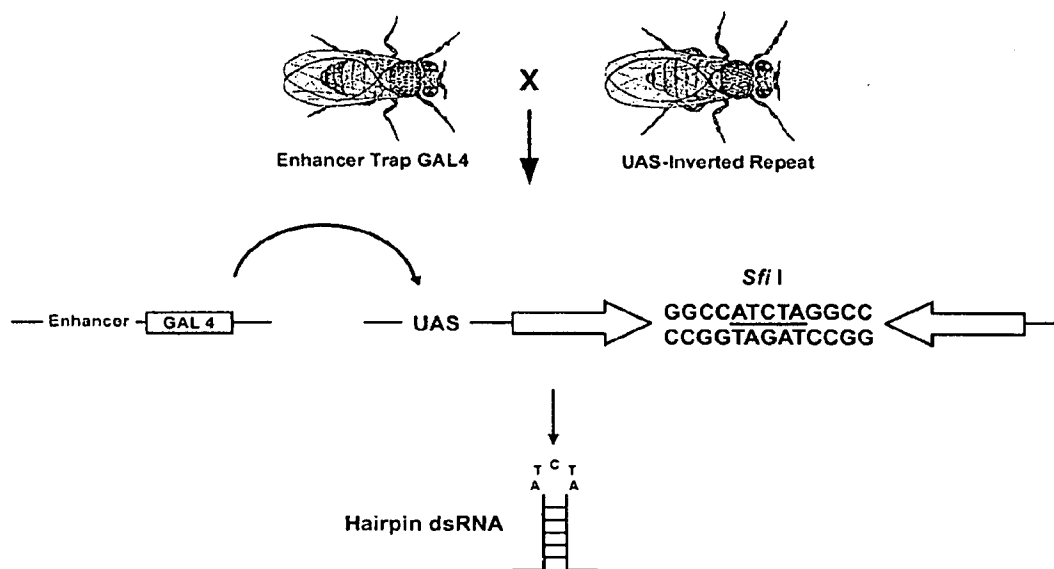


Fig. 1. First-generation transgenic RNAi. Inverted repeats are made by head–head or tail–tail ligation through a *Sfi*I site, which is introduced at one end of each repeat. The inverted repeat is placed downstream of the upstream activating sequence (UAS) promoter, and transgenic lines are made. When these UAS lines are crossed to GAL4 driver lines, the F1 heterozygotes contain both GAL4 and UAS genes [13]. Tissue-specific expression of the inverted repeats by GAL4 protein produces hairpin-loop RNA that is competent to induce RNAi in *Drosophila*.



crossed to a target UAS line carrying a single target P element inserted at a unique and random position in the genome. The target element carries a GAL4-responsive UAS enhancer, and progeny that contain both GAL4 and UAS elements express the IR sequence in cells expressing GAL4. Phenotypes due to the presence of hairpin-loop RNA in these cells can then be scored directly in flies. The RNAi system takes advantage of two very useful techniques in *Drosophila*: P element transformation and the modular GAL4/UAS system. The modular design makes analysis by RNAi flexible since hairpin-loop RNA can be produced in any spatial or temporal pattern. Moreover, RNAi is conditional, dependent on the presence of both UAS and GAL4 elements in the same individual. Thus, RNAi that might induce lethal or sterile phenotypes is conditionally generated in selected flies, and stably inherited *Drosophila* lines carrying the UAS element alone can be propagated without deleterious RNAi effect.

One feature of the target element that was critical for the success of this method was that the IR was stable in the *Drosophila* genome. This appears to be the case since UAS lines have been maintained in our lab stocks for 2 years without loss of RNAi potency when crossed with GAL4 driver lines. However, two other features of the method have proved to be more problematic. First, RNAi silencing is frequently variable, with only a fraction of treated animals exhibiting complete silencing. This partial effect is also observed at the level of target mRNA abundance in that a pooled population of treated animals might exhibit at most a fourfold reduction in mRNA levels. Thus, treated individuals have a spectrum of RNAi-induced phenotypes, which makes interpretation of gene function somewhat difficult. Moreover, there is frequently a variation in the strength of RNAi effects between different transformant lines carrying the same IR transgene. This is likely due to influence of nearby chromosomal modulation of transgene expression that depends on the point of transgene insertion. Since RNAi silencing is not complete, weak or strong IR expression translates to a corresponding weak or strong silencing effect. A second problem with this first-generation RNAi vector has been the variable success in cloning IRs from different genes. Some IRs are easily cloned even into *shcBC<sup>+</sup>* bacterial strains. However, some IRs have proved recalcitrant to cloning in any strain or any plasmid. To date, we have been unable to predict which sequences will produce problems when cloned as IRs. This makes transgene production a somewhat empirical trial-by-error process.

### 3. Transgenic RNAi with inverted exon repeats

The problems with the first-generation vector inspired us to devise a new approach to produce hairpin

RNA in vivo. It was reported that intron-spliced hairpin RNA can induce gene silencing in plants more efficiently than standard hairpin-loop RNA [15]. In a sense, the inverted repeats are structural and functional exons. The nonpalindromic intron sequence may also provide stability to the DNA construct with inverted repeats in bacteria. This led us to test an RNAi construct containing inverted repeats separated by an intron sequence, from which loopless hairpin dsRNA is predicted to be produced following splicing in *Drosophila* (Fig. 2). As a proof-of-principle demonstration of its effectiveness, we decided to test the approach on silencing the *white* gene.

The *Drosophila white* gene encodes an ABC transporter involved in localizing pigments to eye pigment granules [16]. The *white* gene was chosen because expression can be easily monitored phenotypically by changes in eye color. A *white<sup>+</sup>* eye is dark red in color while the eye of a null *white* mutant is completely white in color (Fig. 3). Since cloned variants of *white* are routinely used in *Drosophila* transformation vectors as the selectable marker for transformation of *white* mutant flies, we adopted an opposing transformation strategy. That is, we constructed a transformation vector with a *white* IR but no independent marker gene for selecting transformants. We then transformed *white<sup>+</sup>* flies with the vector and selected transformants that had a *white* loss-of-function phenotype. If an inserted *white* IR transgene successfully silenced its endogenous target gene, the transformant would be white-eyed.

The 74-nucleotide second intron of the *white* gene bears all features of a consensus *Drosophila* intron, and it was found to efficiently splice in *Drosophila* embryonic extracts in vitro [17,18]. Since *white* is normally not expressed in embryos, this result indicates that the intron can be spliced in heterologous tissues. Thus, we chose the second intron to separate inverted repeats of *white* coding sequence in our model transgene.

The 629-bp third exon of *white* was chosen to be the inverted sequence in the transgene that would mediate the RNAi effect. The third exon was amplified by PCR with unique *Pst*I and *Eco*RI sites, and it was ligated in inverted orientation upstream of a 703-bp fragment containing the *white* second intron and third exon (Fig. 2). The tail-to-tail repeat was placed into the pGMR transformation vector plasmid [19]. pGMR drives expression of transgenes specifically in the developing and adult compound eye by virtue of the eye-specific GMR promoter. This ensured that the *white* IR transgene would be expressed only in the same cells that normally express the endogenous target *white* gene. The *Drosophila* consensus sequence for a 5' splice site is AG|GTRAGT, where | designates the splice site and R indicates A or G [17]. It is noteworthy that the ligation between the two DNA fragments through the *Pst*I site does not change the consensus sequence required for

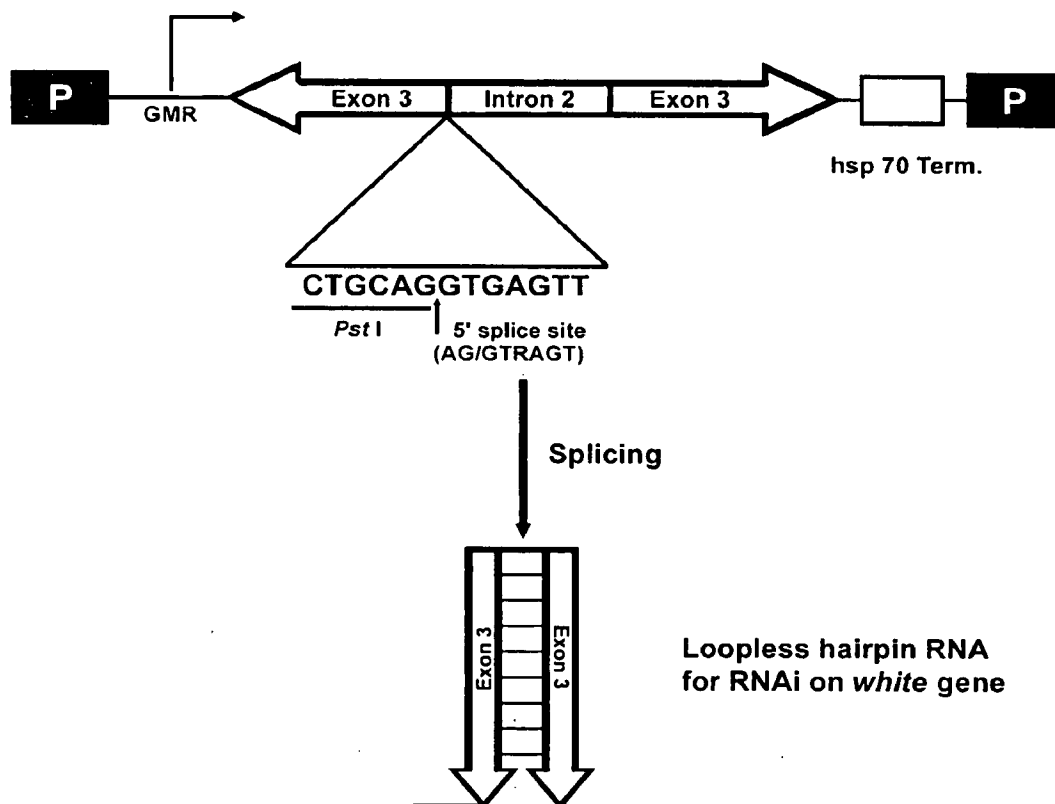


Fig. 2. Scheme for the generation of transgenic RNAi against the *Drosophila white* gene by intron-spliced hairpin RNA. Inverted repeats corresponding to the third exon of the *white* gene and separated by the second intron of the same gene were placed into the pGMR transformation vector. A fragment containing the *white* second intron and third exon was ligated to a fragment containing the inverted *white* third exon to generate a *Pst*I site at the point of ligation. Note that the *Pst*I site is coincident with the 5' splice site but does not disrupt sequences necessary for 5' splice site function. The *Drosophila* consensus sequence for a 5' splice site is shown in parentheses, in which / designates the splice site and R indicates A or G. The transgene is under transcriptional control of the GMR enhancer, which is exclusively active in the developing and adult retinal tissue that also expresses the endogenous *white* gene.

5' splice site recognition (Fig. 2). Since we intended to induce RNAi on the endogenous *Drosophila white* gene, an *Xho*I–*Nsi*I fragment corresponding to the pGMR *white* marker gene was deleted from pGMR.

Although we transformed ligation products including inverted repeats into the SURE strain (Stratagene) of *E. coli* to maximize the stability of the DNA, the repeats were also stable in a DH5 $\alpha$  strain, possibly as a result of the short *white* second intron sequence contributing to the stability of the inverted-repeat sequences. In contrast, attempts at cloning an IR of the *white* third exon separated by a *Sfi*I spacer into plasmids was repeatedly unsuccessful in a variety of host bacterial strains.

The pGMR-derived plasmid containing the DNA fragment for intron-spliced *white* hairpin RNA was introduced into the germ line of CantonS flies by P element transformation [14]. From approximately 1500 injected animals, eight independent transformant lines that exhibited a *white* loss-of-function phenotype were established. This transformation frequency is within an order of magnitude of the average transformation fre-

quency using a standard P element vector [14], which suggests that *white* RNAi from the IR transgene acts as a reliable marker for transformation. All eight transformant lines exhibited a yellow to pale-yellow eye color phenotype with one copy of the transgene (Fig. 3). Moreover, all individual flies from each line exhibited a uniform eye color phenotype, indicating strong penetrance and expressivity of the RNAi effect. Only females were compared to avoid any effect related to dosage compensation of the transgene. No additional or abnormal phenotypes were observed in silenced individuals, indicating that silencing was specific. The effect was stably maintained over each adult's lifetime, and silencing has been maintained over the many generations that these lines have so far been kept. Transformant adults bearing two copies of the transgene had an eye color indistinguishable from that of *white* null mutants (Fig. 3). Levels of *white* mRNA on a Northern blot were reduced in two transformant lines tested compared to wild type, to a degree consistent with their eye color phenotypes (data not shown). In conclusion, the

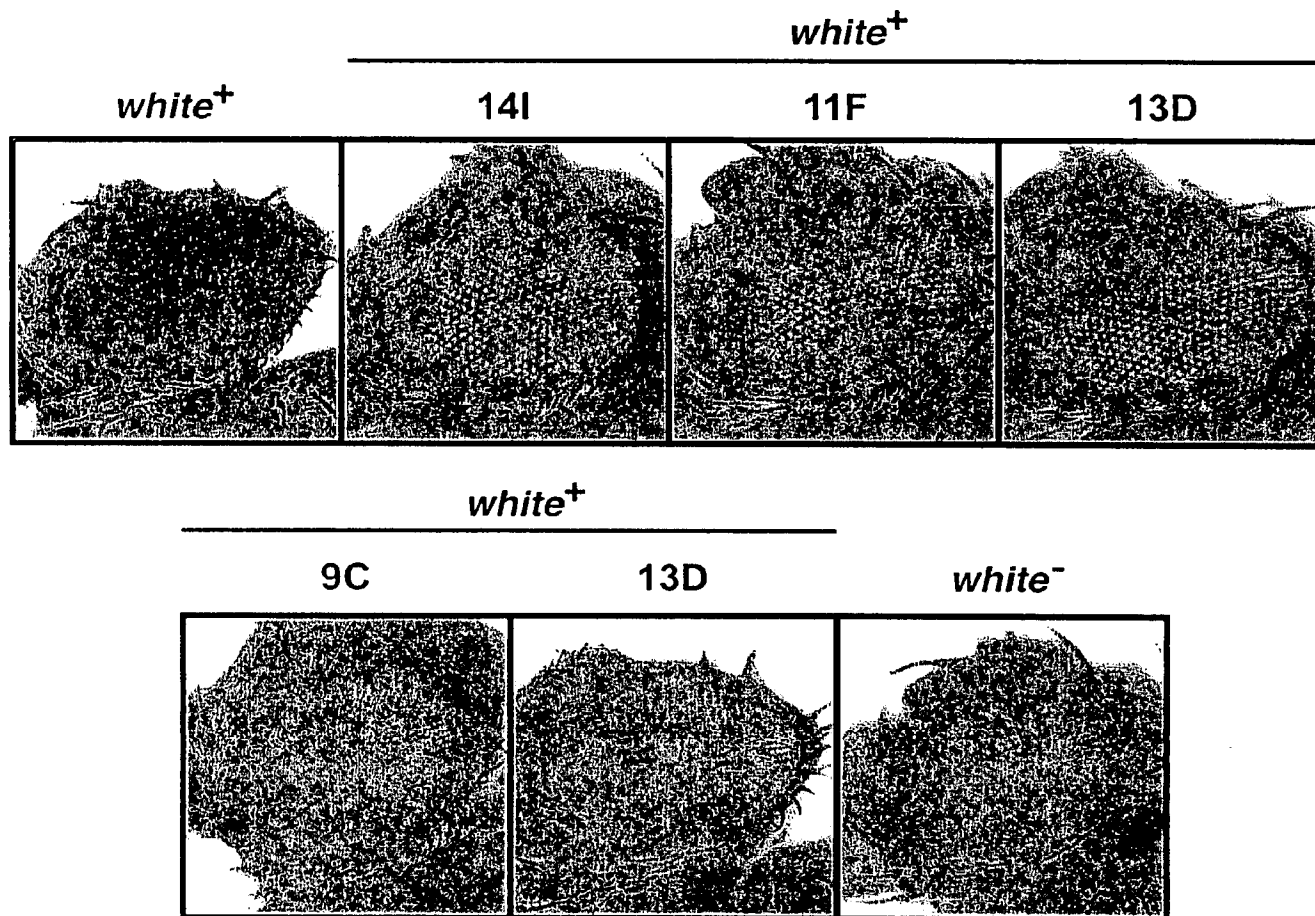


Fig. 3. Eye color phenotypes of female adults (3–5 days of age) that carry the *white* intron–hairpin GMR transgene. The top left shows a parental CantonS (wild-type *white*) fly. The lower right shows a *w<sup>1118</sup>* null mutant for the *white* gene. A P[GMR-*whiteIR*] transformant line designated 13D has the transgene on the X chromosome of the parental CantonS strain, whereas the 9C, 11F, and 14I lines have the transgene on the third chromosome of CantonS. The eye colors of transformants bearing a single copy of the transgene is shown in the top. The bottom shows eye colors of transformants bearing two copies of the transgene.

inverted exon repeat of *white* effectively silences the expression of the endogenous *white* gene in vivo.

#### 4. A modular and multipurpose transgenic RNAi vector

Since the RNAi construct bearing an intron strongly inhibited *white* gene expression, we adapted this method to create an all-purpose RNAi vector that employs spliced hairpin RNA. The vector is derived from the pUAST transformation plasmid. This then offers the advantages of the GAL4/UAS modular expression system, as outlined earlier. We constructed the vector (pWIZ, for *white* intron zipper) into which gene fragments can be subcloned upstream and downstream of the 74-nucleotide *white* intron (Fig. 4). The intron is flanked by *EcoRI*, *BglII*, *NotI*, *XhoI*, *SpeI*, and *AvrII* sites on the 5' side and by *NheI*, *MluI*, and *XbaI* sites on the 3' side. The entire cassette is downstream of the UAS

enhancer–promoter and upstream of the SV40 transcription termination site. The *AvrII* and *NheI* sites in pWIZ conform to the consensus sequences for 5' and 3' splice sites, respectively. Thus, any DNA fragment inserted into the *AvrII* or *NheI* site is fully competent to be spliced as an exon. Moreover, the *SpeI*, *AvrII*, *NheI*, and *XbaI* sites are unique in pWIZ, providing convenient cloning sites for gene fragments.

To construct an IR transgene using pWIZ, a DNA fragment corresponding to the gene of interest is inserted twice into pWIZ, with inserts in opposite orientations on each side of the intron (Fig. 5). The simplest means to insert the DNA is as a PCR fragment. The system is designed so that a single PCR fragment derived from only two PCR primers can be inserted on each side of the intron. This is because *SpeI*, *AvrII*, *NheI*, and *XbaI* sites are all ligation-compatible with each other. Consequently, restriction sites compatible with *AvrII* and *NheI* sites should be placed in the PCR

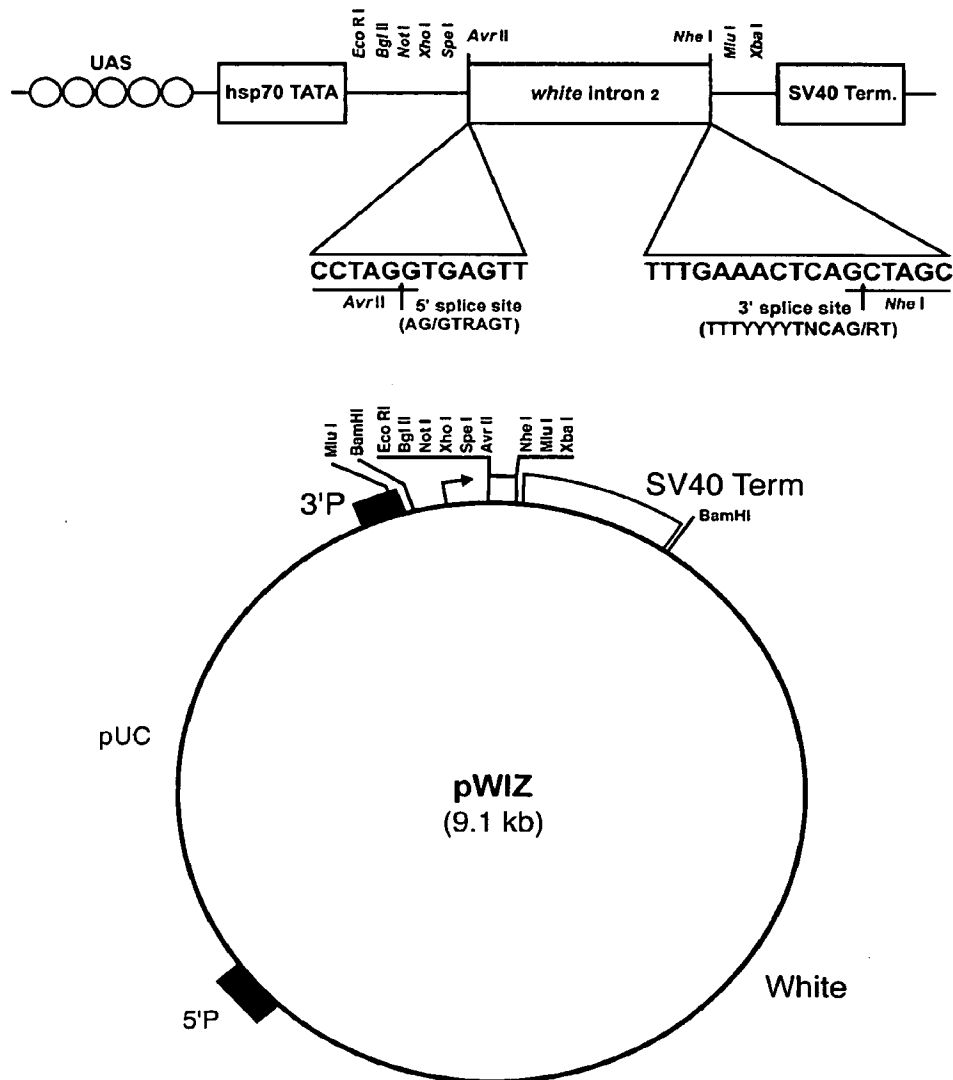


Fig. 4. Schematic representation of the pWIZ vector. The pWIZ vector was constructed by placing the 74-bp second intron of the *white* gene into the pUAST transformation vector [13]. The intron is flanked by unique *EcoRI*, *BglII*, *NotI*, *XhoI*, *SpeI*, and *AvrII* sites on the 5' side and *NheI* and *XbaI* sites on the 3' side to facilitate cloning. The sequences at the junction of the 5' and 3' splice sites in the vector are highlighted, and arrows indicate the 5' and 3' splice sites. The consensus sequences for 5' and 3' splicing are shown in parentheses: /, the splice site; R, purine; Y, pyrimidine; N, any base. Below is shown a restriction map of the pWIZ plasmid.

primers at their 5' ends. The resulting PCR product will then have *AvrII*- and *NheI*-compatible ends after appropriate restriction digestion. The RNAi construct is made by two sequential insertions of the same PCR product into the *AvrII* and *NheI* sites of pWIZ (Fig. 5). For efficient digestion, we add an extra 4 nucleotides to the 5' side of each primer restriction site. The size of amplified DNA ranges from 500 to 700 bp. Most important, we ensure that the sequence does not contain any internal restriction sites used in the PCR primers, nor should the fragment have sequences in either sense or antisense orientation that match either 5' or 3' consensus splice sites. This latter aspect is important to

prevent cryptic splicing from disrupting hairpin RNA formation. The PCR product is digested with the appropriate enzyme and ligated into the *AvrII* site of pWIZ. After a clone with the desired orientation of the insert is selected by restriction mapping, the same PCR product is ligated into the *NheI* site of the pWIZ derivative, and recombinants with the insert in opposite orientation to the first are screened and selected.

We have made five transgenic RNAi constructs for genes under study in our laboratory using the pWIZ vector. All of these constructs are stable as inverted repeats in *E. coli* strains such as SURE cells. Moreover, they have been introduced into the *Drosophila* genome

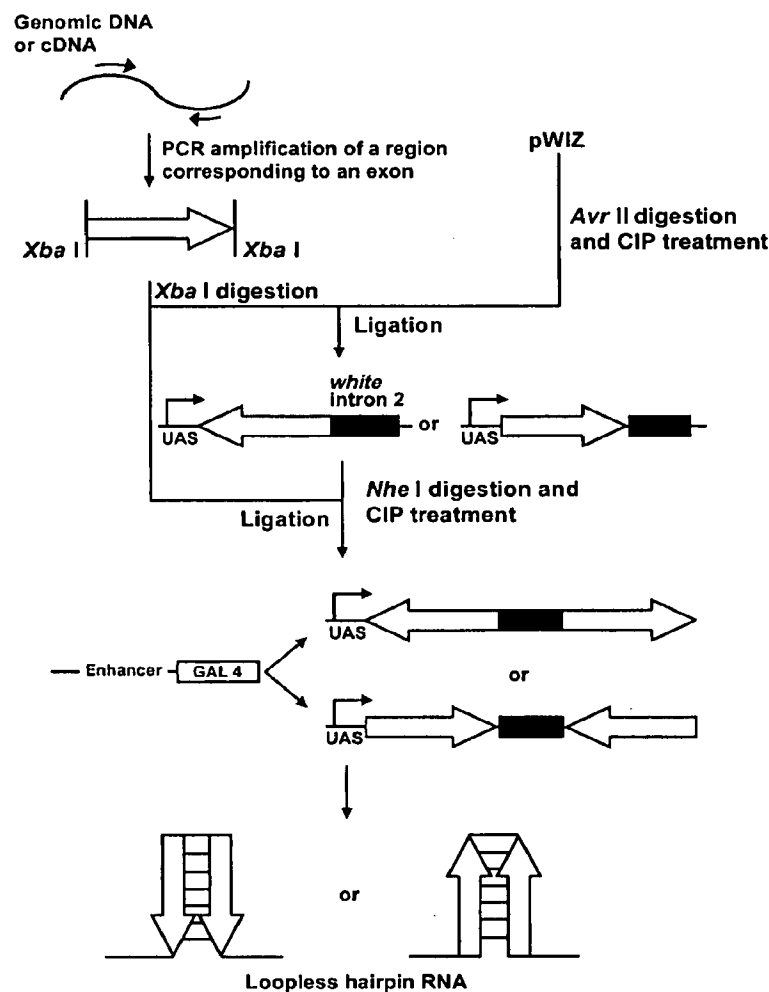


Fig. 5. A typical procedure for making a RNAi construct using the pWIZ vector. A DNA fragment corresponding to a *Drosophila* target gene is amplified by PCR. A restriction site (*SpeI*, *AvrII*, *NheI*, or *XbaI*) compatible with *AvrII* and *NheI* should be present at the 5' end of each PCR primer. The fragment can correspond to part of an exon, a complete exon, or several contiguous exons. Several criteria should be used in choosing the fragment: it should be 500 to 700 bp in length, it should not have internal restriction sites corresponding to the PCR primer sites, and it should not have sequences in either sense or antisense orientation that match a 5' or 3' consensus splice site. This latter aspect is critical to prevent cryptic splice sites from disrupting proper splicing of the hairpin RNA. As shown, an *XbaI* site is generated at each end of a PCR-amplified exon for cloning the PCR product. The PCR product is inserted twice by two ligation steps into the *AvrII* and *NheI* sites of pWIZ. CIP, calf intestinal phosphatase used to dephosphorylate the 5' ends of vector DNA prior to ligation. Recombinants are selected in the desired orientation, such that after the second ligation step, inserts are in opposite orientation on either side of the *white* intron. IRs that are head-head or tail-tail repeats might be used depending upon whether cryptic splice sites are present in the constructs. Transformation follows to generate stable transgenic lines carrying the WIZ gene. Upon mating of transgenic animals harboring the WIZ gene with animals carrying tissue- or cell-specific GAL4 drivers, the F1 progeny produce loopless hairpin RNA. This induces RNAi against target genes in tissue- and cell-specific patterns.

by P element transformation, and all constructs transformed efficiently to give stable lines.

## 5. Concluding remarks

In conclusion, we have developed transgenic RNAi in *Drosophila* that can be applied to many developmental and physiological processes. Hairpin RNA produced from a transgene composed of inverted repeats can spe-

cifically silence gene expression in *Drosophila*. The presence of a spacer between the inverted repeats makes for easier cloning but is offset by a weaker silencing activity in vivo [8,20]. In plants, using a functional intron as the spacer between inverted repeats strongly enhanced silencing activity of the RNAi transgene [15]. We have shown that using a functional intron as a spacer between inverted repeats produces strong and uniform RNAi silencing in *Drosophila*. A similar observation has been recently noted in *Drosophila* when inverted repeats

composed of cDNA–genomic DNA hybrids are separated by functional introns [21]. We have also described a multifunctional RNAi transformation vector (pWIZ) containing an intron spacer that makes RNAi simple to perform for the following reasons. A single PCR fragment of a gene is sufficient to construct a targeting vector; the inverted repeat sequence need not have splice sites present since they are provided by pWIZ; splice sites are preserved when the repeat fragments are inserted; the intron spacer provides stability to the inverted repeats when the plasmid is replicated in *E. coli*. Once the vector is transformed into *Drosophila*, it is conditionally quiescent until crossed with GAL4-expressing lines. Many useful GAL4-expressing lines are available, making the RNAi approach adaptable for most studies of *Drosophila*. This method is likely to be very useful for analyzing the function of the many *Drosophila* genes for which no loss-of-function mutations are available. Finally, the method provides a powerful tool to create loss-of-function phenotypes in a manner conditional for particular tissues and developmental times.

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# The Cotton *ACTIN1* Gene Is Functionally Expressed in Fibers and Participates in Fiber Elongation

Xue-Bao Li,<sup>a,b</sup> Xiao-Ping Fan,<sup>b</sup> Xiu-Lan Wang,<sup>a</sup> Lin Cai,<sup>b</sup> and Wei-Cai Yang<sup>b,c,1</sup>

<sup>a</sup>College of Life Sciences, Central China Normal University, Wuhan 430079, China

<sup>b</sup>Temasek Life Sciences Laboratory, National University of Singapore, Singapore 117604

<sup>c</sup>Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100080, China

Single-celled cotton fiber (*Gossypium hirsutum*) provides a unique experimental system to study cell elongation. To investigate the role of the actin cytoskeleton during fiber development, 15 *G. hirsutum* *ACTIN* (*GhACT*) cDNA clones were characterized. RNA gel blot and real-time RT-PCR analysis revealed that *GhACT* genes are differentially expressed in different tissues and can be classified into four groups. One group, represented by *GhACT1*, is expressed predominantly in fiber cells and was studied in detail. A 0.8-kb *GhACT1* promoter sufficient to confirm its fiber-specific expression was identified. RNA interference of *GhACT1* caused significant reduction of its mRNA and protein levels and disrupted the actin cytoskeleton network in fibers. No defined actin network was observed in these fibers and, consequently, fiber elongation was inhibited. Our results suggested that *GhACT1* plays an important role in fiber elongation but not fiber initiation.

## INTRODUCTION

Actin cytoskeleton plays an important role in cell morphogenesis in plants as demonstrated by pharmacological, biochemical, and genetic studies (Kost and Chua, 2002; Mathur and Hülskamp, 2002). The actin cytoskeleton may be involved in the transportation of organelles and vesicles carrying membranes and cell wall components to the site of cell growth as in root hairs, trichome cells, and pollen tubes. Therefore, the actin cytoskeleton is essential for cell elongation and tip growth. Disruption of the actin cytoskeleton during trichome development by actin-interacting drugs resulted in randomly distorted trichomes with unextended branches (Mathur et al., 1999; Szymanski et al., 1999). Similarly, inhibition of F-actin elongation blocked the initiation of polar growth and elongation of root hairs (Miller et al., 1999). Furthermore, reduction in actin arrays resulted in dramatic reduction of root hair length and caused severe bulges in the *actin2* (*act2*) mutant and serious retardation of root growth in the *act7* mutant in *Arabidopsis thaliana* (Gilliland et al., 2002, 2003). Misexpression of the reproductive *ACT11* gene in vegetative tissues of *Arabidopsis* altered morphology of most organs in plants because of its effects on the proportion of different actin isoforms (Kandasamy et al., 2002). In polarized elongating cell types, such as root hairs and trichomes, it is believed that long F-actin cables oriented longitudinally throughout the shank and

subapical, and net-axially aligned fine F-actins are essential for the intracellular trafficking of organelles and secretory vesicles to the growing apical region to deliver new membranous and cell wall materials (Mathur et al., 1999; Miller et al., 1999; Szymanski et al., 1999; Baluska et al., 2000; Hepler et al., 2001; Chueng et al., 2002). The unstable dynamic F-actin cytoskeleton also plays a role in localized expansion of root hairs and trichome cells (Ketelaar et al., 2003; Mathur, et al., 2003a).

The actin cytoskeleton controls polar cell growth through its interaction with several actin binding proteins, such as actin depolarizing factor (Dong et al., 2001; Chen et al., 2002), profilin (Clarke et al., 1998), Rho family GTPase (Yang, 1998; Chueng et al., 2002; Fu et al., 2002), and the calcium signaling pathway (Malhó, 1998; Franklin-Tong, 1999; Li et al., 1999). The effective regulation of actin turnover by actin regulators may be critical for pollen tube growth (Chen et al., 2002, 2003) and for polar cell expansion in cell types other than root hair and trichome (Fu et al., 2002). Recent studies showed genetically that the actin cytoskeleton by interacting with the ARP2/ARP3 complex plays a pivotal role in controlling cell shape of trichome cells and several other cell types in *Arabidopsis* (Mathur et al., 2003a, 2003b). In cotton (*Gossypium hirsutum*), F-actin has been implicated in regulating microtubule orientation during fiber development shown by in vitro drug studies (Seagull, 1990). However, the role of the actin cytoskeleton in cotton fiber cell development remains largely unknown.

Actins in plants are encoded by a multigene family that comprises dozens or even hundreds of actin genes. In *Arabidopsis*, the actin gene family contains 10 distinct members, of which eight are functional genes and two are pseudogenes (McDowell et al., 1996). In other plant species, the actin gene family also appears to have dozens of members (Baird and Meagher, 1987; Thangavelu et al., 1993; Meagher and Williamson, 1994). Studies on actin sequences revealed that structural and

<sup>1</sup>To whom correspondence should be addressed. E-mail wcyang@genetics.ac.cn; fax 86-10-62551272.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) are: Xue-Bao Li (xbli@mail.ccnu.edu.cn) and Wei-Cai Yang (wcyang@genetics.sc.cn). Article, publication date, and citation information can be found at www.plantcell.org/cgi/dol/10.1105/tpc.104.029629.

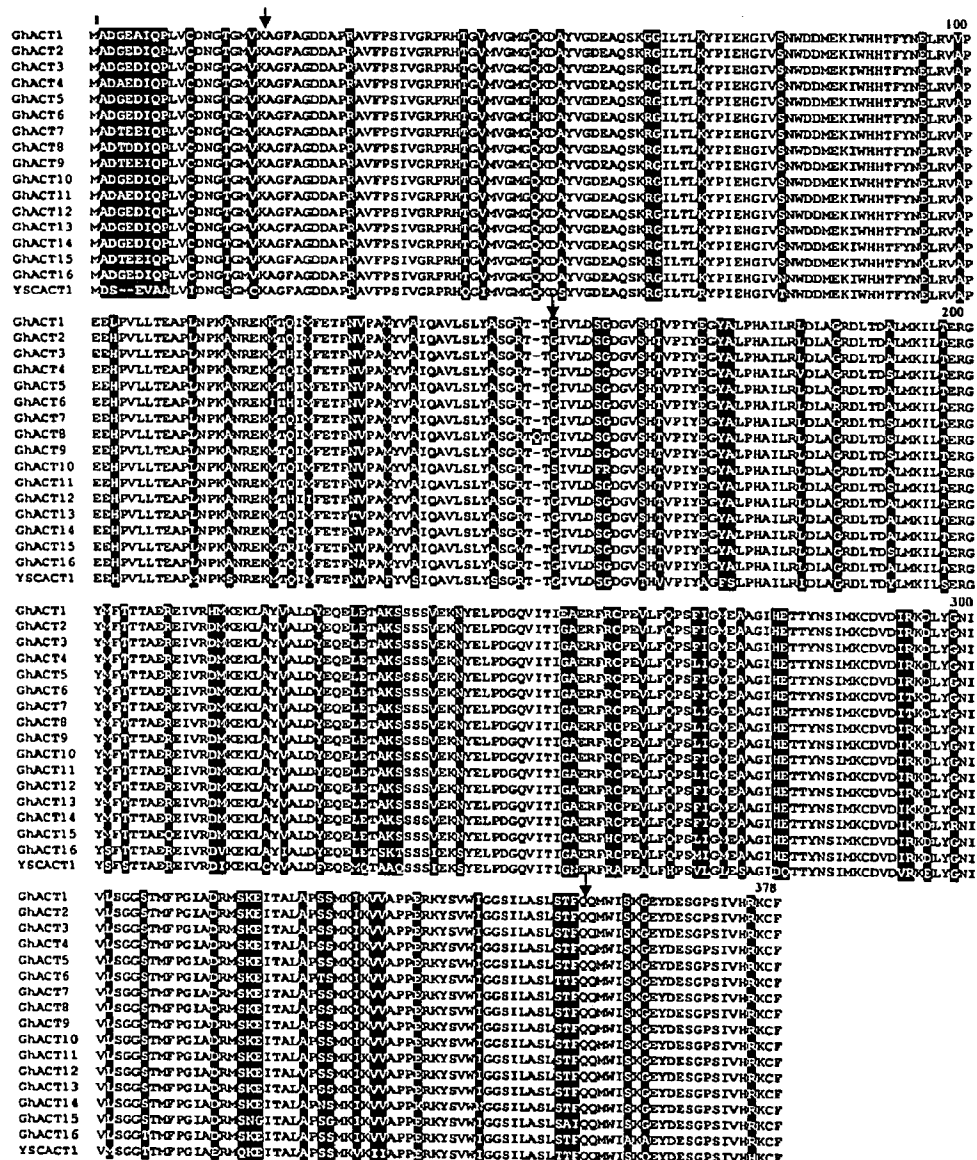


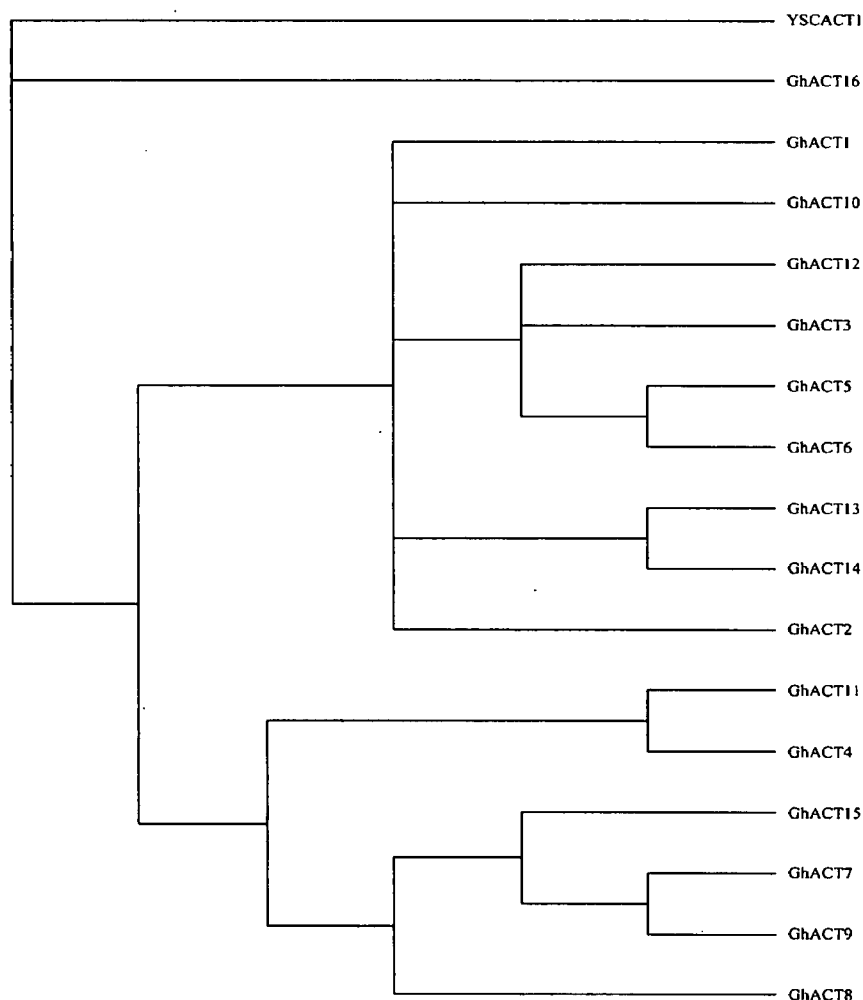
Figure 1. Comparison of the Predicted Amino Acid Sequences of Cotton *GhACT* Genes.

Multiple alignment of amino acid sequences of 16 cotton *GhACT* genes and yeast *YSCACT1*. Amino acid substitutions are highlighted in black. Arrows indicate the positions of the three introns in cotton *GhACT* genes. *GhACT1* to *GhACT15* were from this work; *GhACT16* is a putative actin derived from a genomic sequence in GenBank (accession number AF059484).

functional divergence occurred within the gene family during evolution (McDowell et al., 1996; Meagher et al., 1999a). Members of the actin gene family are divergent and differentially expressed during plant development. Arabidopsis contains two major actin gene classes: a vegetative class that is expressed predominantly in leaves, stems, roots, petals, and sepals and

a reproductive class that is strongly expressed in pollens, ovules, and embryonic tissues (McDowell et al., 1996; Kandasamy et al., 1999). The soybean (*Glycine max*) actin gene family includes at least three divergent classes:  $\mu$ -,  $\kappa$ -, and  $\lambda$ -actin. The  $\mu$ -actin transcripts are differentially accumulated in leaves, roots, and hypocotyls. The  $\kappa$ - and  $\lambda$ -actin proteins are preferentially localized





**Figure 2.** Phylogenetic Relationships of Cotton Actins.

The rooted gene tree shown is based on majority-rule consensus from 500 bootstrap replicates and resulted from heuristic searching in PAUP 4.0, based on amino acid sequences of the *GhACT* genes. Cotton GhACT1 to GhACT15 actins were from this work; GhACT16 is a putative actin derived from a genomic sequence in GenBank (accession number AF059484); YSCACT1 is a yeast actin (accession number L00026) used as an outgroup.

in roots (McLean et al., 1990). In other plant species, such as rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*), actin genes also appear to be expressed in a tissue-specific manner (McElroy et al., 1990; Thangavelu et al., 1993). Although actin genes in a few plant species such as *Arabidopsis* have been well characterized, our knowledge of cotton actin genes, especially its role in fiber development, needs to be explored.

Cotton fibers, as a premier natural fiber and extensively used in the textile industry, are derived from epidermal cells of the reproductive organ, the ovule. Approximately 30% of the ovule epidermal cells elongate and develop into single-celled fibers at anthesis. Each fiber is perhaps the longest single cell in higher plants. Its elongation rate and the final length attained are far

above that of common plant cells (Cosgrove, 1997). Fiber development is a highly regulated process involving four sequential stages: fiber initiation, primary cell wall formation, secondary cell wall formation, and maturation (Basra and Malik, 1984). Thus, the cotton fiber represents a unique experimental system for studying the control of cell elongation without the complication of cell division and multicellular development (Ruan et al., 2001). The study on fiber development not only provides the basic understanding of cell differentiation and elongation, but also identifies potential target genes for genetic manipulation of cotton fiber. Here, we reported the identification and characterization of the actin gene family in cotton and explored its role in fiber development using RNA interference (RNAi) technology.

Table 1. Primers Used in Gene-Specific RT-PCR of *GhACT* Genes

Genes	Primers
<i>GhACT1</i>	5'-CCCTTGAATATTAATAAATAAAAAATA-3' 5'-TTGTGCTCAGTGGGGTTCAACC-3'
<i>GhACT2</i>	5'-TGCCCGGAAGTCCTCTCCAG-3' 5'-ATTTTCCCAGAGTTTGACCGCGC-3'
<i>GhACT3</i>	5'-CCCTTGAATATTAATAAATAAGCAC-3' 5'-TTGTGCTCAGTGGGGTTCAACT-3'
<i>GhACT4</i>	5'-GGGGGAGCCTTGAATATGAAATTG-3' 5'-TTGTGCTCAGTGGGGTTCAACC-3'
<i>GhACT5</i>	5'-ATTTTCCCAGAGTTTGACCGCGC-3' 5'-TGCCCGGAAGTCCTCTCCAA-3'
<i>GhACT7</i>	5'-TTAAAGAAATATAAGAAATAAGCATCA-3' 5'-GTATGCCAGTGGTCGACGACA-3'
<i>GhACT8</i>	5'-TTAAAGAAATATAAGAAATAAGCATCA-3' 5'-GTATGCCAGTGGTCGACGACG-3'
<i>GhACT9</i>	5'-ATCTTCAACATAAAAGATCATCCCACT-3' 5'-GATCTATCTTGGCATCACTCAGCA-3'
<i>GhACT10</i>	5'-AACCAGATATTAATAATAATTTCCGTAG-3' 5'-GGGAAATTGTCCGTGACATGAAG-3'
<i>GhACT11</i>	5'-ACAATAGCTATTGACATTATGTTTGC-3' 5'-TTGTGCTCAGTGGGGTTCAACT-3'
<i>GhACT12</i>	5'-AACCAGATATTAATAATAATTTCCGTAG-3' 5'-GGGAAATTGTCCGTGACATGAAA-3'
<i>GhACT13</i>	5'-CCCTTGAATATTAATAAATAAGCAC-3' 5'-TTGTGCTCAGTGGGGTTCAACC-3'
<i>GhACT14</i>	5'-AACCAGATATTAATAATAATTTCCGTAA-3' 5'-ATTGGAGCTGAGAGATTCCGTTG-3'
<i>GhACT15</i>	5'-ATCTTCAACATAAAAGATCATCCCACT-3' 5'-GATCTATCTTGGCATCACTCAGCG-3'
<i>GhUBI</i>	5'-CTGAATCTTCGCTTTCACGTTATC-3' 5'-GGGATGCAATCTTCGTGAAAC-3'

The efficiency of each primer pair was detected using *GhACT* cDNA clones as standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair.

## RESULTS

### Isolation and Characterization of *GhACT* cDNAs

To isolate genes involved in cotton fiber development, we have randomly sequenced >300 cDNA clones from a fiber cDNA library (Li et al., 2002). Clones, including an actin cDNA, likely involved in cell elongation were chosen for further study. Using the actin cDNA clone as probe, we further isolated 15 unique actin cDNAs (designated *GhACT* genes; accession numbers in GenBank, AY305723 to AY305737) from a cotton cDNA library. Sequence analysis predicted that all *GhACT* genes, except *GhACT8*, encode a 377-amino acid polypeptide. The *GhACT8* encodes an actin containing 378 amino acid residues with a Gln insertion at position 151 (Figure 1). The *GhACT* genes share high sequence homology at nucleotide level (70 to 97% identity) in the coding region and at the amino acid level (93 to 99% identity). There is only 1 to 7% substitution rate at amino acid level compared with each other (Figure 1). In total, 21 charged substitutions occurring at 14 charged positions were present in *GhACTs*. Among them, charged amino acids were exclusively

substituted with uncharged residues at six locations (Arg/Gly, Thr or Gln, Asp/Ala, Glu/Gly, His/Leu, or Lys/Trp) and were only substituted by a synonymous charged amino acid at other positions. The charged amino acids at residues 6 and 292 were substituted by either a charged amino acid or an uncharged residue (Figure 1), suggesting that these positions may not be important for actin structure. While at residue 123, both charged and polar uncharged amino acids were present in *GhACTs*. In addition, 11 uncharged amino acids at six positions were substituted by a charged residue. Often in this case, Gln was substituted by a His and Gly replaced by an Arg. Intriguingly, most nonsynonymous substitutions occur only in *GhACT1* protein. For example, positively charged amino acids were substituted by a nonpolar, uncharged amino acid at positions 64 and 103. On the other hand, nonpolar amino acids were replaced by positively charged and negatively charged polar residues at positions 121 and 253, respectively. At position 213, the negatively charged Asp was substituted by a positively charged His, suggesting that *GhACT1* may have a different structure and function than other *GhACT* variants.

Phylogenetic analysis on amino acid sequences showed that the 16 *GhACTs* available could be divided into nine subgroups (Figure 2). Among them, five subgroups contain only a single member, and the remaining four subgroups have two to four members. Each of *GhACT1*, *GhACT2*, *GhACT8*, *GhACT10*, and *GhACT16* forms an independent clade, suggesting that these *GhACTs* diverged early during evolution, whereas *GhACT3*, *GhACT5*, *GhACT6*, and *GhACT12* together form a single branch, indicating that divergence of these genes occurred relatively late.

### *GhACT* Genes Are Differentially Expressed in Different Organs

To identify *GhACT* genes that are preferentially expressed in cotton fibers, the expression patterns of 15 *GhACT* cDNA clones were analyzed by real-time quantitative SYBR-Green RT-PCR using gene-specific primers (Table 1) as described in Methods. The cotton polyubiquitin gene (*GhUBI*; X.B. Li and W.C. Yang, unpublished data) expressed equally in all tissue types with cycle threshold (Ct) values at  $17.52 \pm 0.35$  and was chosen as a standard control to normalize differences in RNA template concentrations. Five out of the fifteen *GhACT* genes are expressed at relatively high levels in fiber cells (Figure 3A). *GhACT2* is expressed at high levels in all tissues compared with other *GhACTs*, and its expression level reaches a relative value of 17 in fibers as compared with ~5 in other tissue types. For example, *GhACT2* expression in fibers is ~370-fold higher than *GhACT14*. *GhACT1* and *GhACT5* are strongly expressed in fiber and very low in leaf, stem, root, and anther, indicating that they are preferentially expressed in fiber cells. *GhACT4* and *GhACT11* also showed similar expression patterns as *GhACT1* and *GhACT5* in fiber and were moderately expressed in other tissues, whereas the transcripts of other *GhACT* genes are very low, as shown in the small values in the y axis. Overall, *GhACT3*, *GhACT9*, *GhACT10*, and *GhACT12* are expressed at least five-fold less in fibers compared with *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11*. By contrast, the expression of *GhACT7*,

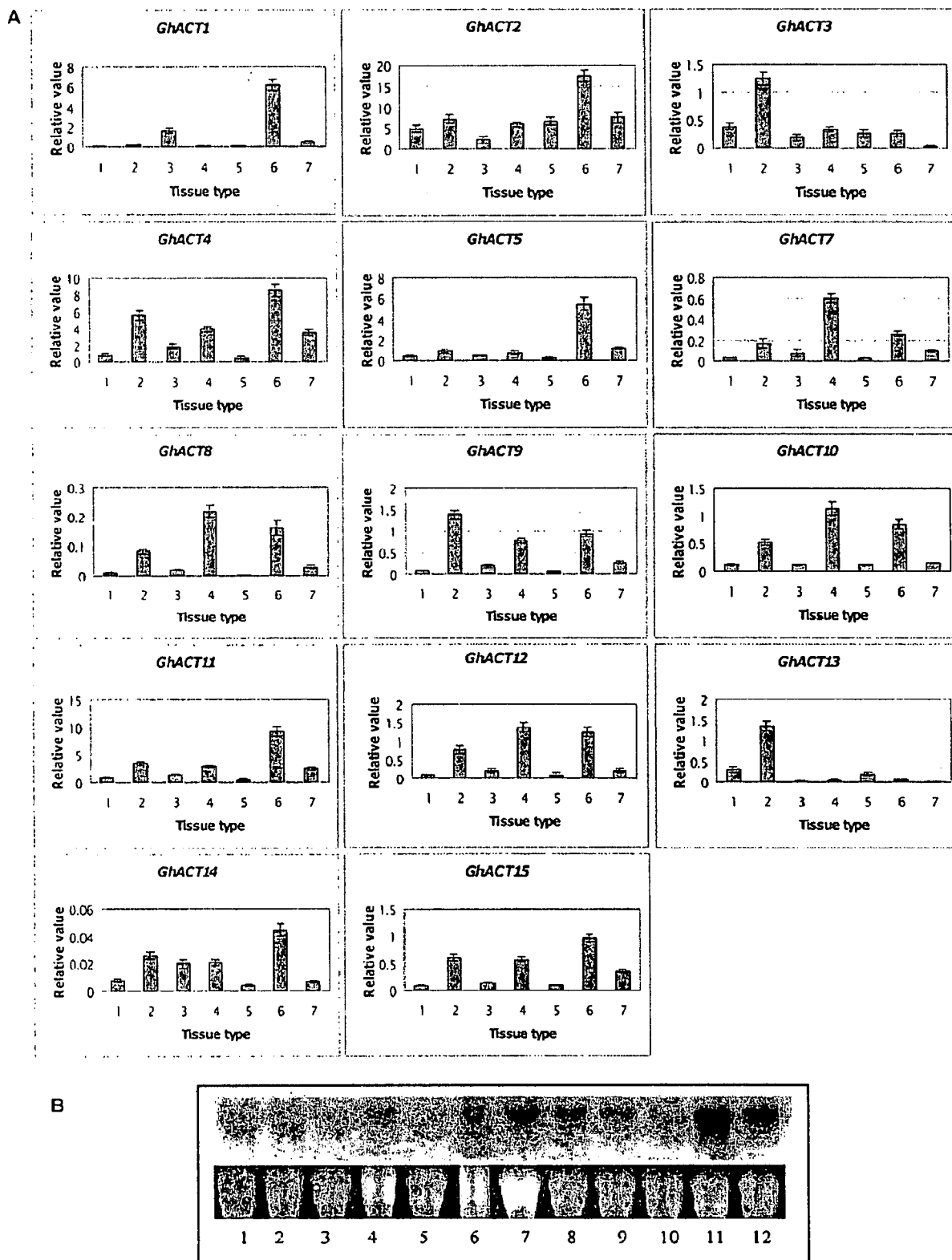


Figure 3. Analyses of Expression of *GhACT* Genes in Cotton Tissues.

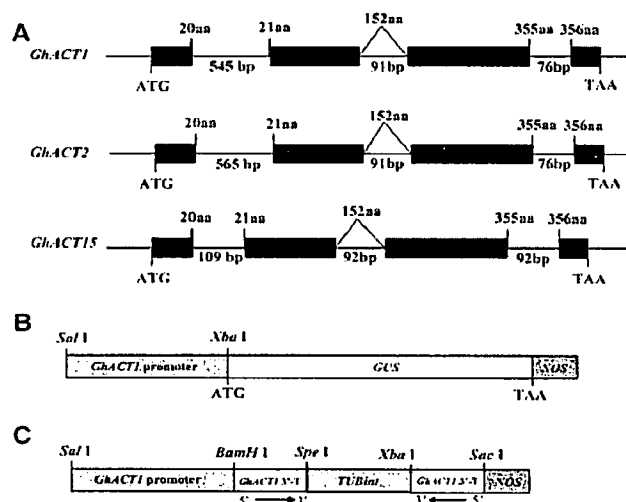
*GhACT8*, *GhACT14*, and *GhACT15* is extremely low if compared with *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11*. Moreover, *GhACT6* expression is not detectable in all the tissues examined. The results of the real-time RT-PCR revealed that the actin genes in cotton were differentially expressed, with *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11* being the predominant forms in fiber cells (Figure 3A).

RNA gel blot analysis, using the 3'-untranslated region (UTR) of *GhACT1* as a probe, further demonstrated that *GhACT1* accumulated at high level in fibers and at a relatively lower level in ovules. The level of *GhACT1* transcripts reached the highest level during 8 to 14 d postanthesis (DPA) and decreased gradually as the ovule developed. At 28 DPA, hardly any transcript was detected. No or very little transcripts were detected in anthers, petals, leaves, and roots (Figure 3B). A moderate level of *GhACT1* was detected in cotyledons. This result further confirmed that the *GhACT1* gene is preferentially expressed, especially in elongation phase in cotton fiber cells.

#### Isolation and Characterization of *GhACT* Genes

Five genomic DNA clones, representing *GhACT1*, *GhACT2*, and *GhACT15* (Figure 4A), were isolated from a cotton genomic library using *GhACT1* cDNA as probe. The isolated *GhACT1* gene is ~3.9 kb in length, including 1.6 kb of the 5' promoter region, 1.8 kb transcribed region, and 0.5 kb 3' downstream sequence. Sequence comparison between cDNA and genomic clones revealed that the three *GhACT* genes all contain four exons and three introns (Figure 4A). The three introns are located exactly at the same positions in all three genes: between amino acid residues 20 and 21, within residue 152, and between residues 355 and 356, respectively. The size and position of introns in *GhACT1* and *GhACT2* are almost identical (Figure 4A). Intron 1 is 545 and 565 bp in length in *GhACT1* and *GhACT2*, respectively, much longer than intron 2 and intron 3. By contrast, intron 1 in *GhACT15* is relatively short, with only 109 bp. The lengths of introns 2 and 3 are similar in all three genes. These data indicate that *GhACT* gene structure is quite conserved in cotton.

To determine the actin gene family copy numbers, cotton genomic DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I, and *Xba*I and subjected to DNA gel blot analysis. There was one major band and one to two weak bands when the 0.8-kb 5' noncoding region of *GhACT1* was used as a probe. The major band represents *GhACT1*, and the weaker bands most likely are due to cross-hybridization with other members of the actin gene family, though the 5' noncoding region was used as probe (Figure 5A). Furthermore, several bands were detected when using the more conserved exon 3 of *GhACT1* as a probe under highly



**Figure 4.** *GhACT* Gene Structure, *GhACT1::GUS*, and *GhACT1* RNAi Construction.

(A) Exons are denoted by black boxes. Introns, 5'-flanking region, and 3'-UTR are denoted by lines. The lengths of the introns in base pairs are indicated. The number at the boundaries of each exon indicates the codon at which the intron is located. The translation initiation and termination codons are shown. aa, amino acids.

(B) The length of the *GhACT1* promoter and cloning sites used for *GhACT1::GUS* fusion are shown.

(C) *GhACT1* RNAi construction.

stringent conditions (Figure 5B). This suggested that there are at least four to eight members of the actin family that share a highly conserved coding region with *GhACT1*, and the remains may diverge earlier during the evolution of the cotton actin gene family.

#### The *GhACT1::β-Glucuronidase* Fusion Gene Is Predominantly Expressed in Cotton Fibers

To characterize the precise expression pattern of *GhACT* genes in cotton fibers, we chose *GhACT1* for further study because it represents *GhACTs* that are expressed preferentially in fibers (Figure 3A) among the three available genomic sequences. A 0.8-kb promoter region of *GhACT1* was subcloned upstream of the  $\beta$ -glucuronidase (*GUS*) reporter gene in pBI101 vector, giving rise to the *GhACT1::GUS* gene (Figure 4B). The *GhACT1::GUS* construct was introduced into cotton cultivar Coker312

**Figure 3.** (continued).

(A) Real-time RT-PCR analysis of expression of *GhACT* genes in cotton tissues. Relative value of *GhACT* gene expression in cotton tissues, including leaf (1), stem (2), cotyledon (3), root (4), anther (5), fiber (6), and petal (7), was shown as percentage of *GhUBI* expression activity (see Methods). (B) RNA gel blot analysis of *GhACT1* transcripts in cotton. Total RNA (20  $\mu$ g/lane) from petal (1), anther (2), leaves (3), cotyledon (4), root (5), ovule (6 to 10) at 4, 8, 14, 21, and 28 DPA, and fiber (11 and 12) at 8 and 14 DPA was fractionated on a 1.2% denaturing agarose gel and transferred onto a nylon membrane (see Methods). Top panel, autoradiograph of RNA hybridization; bottom panel, RNA gel before transfer to membrane showing equal loading of RNAs.

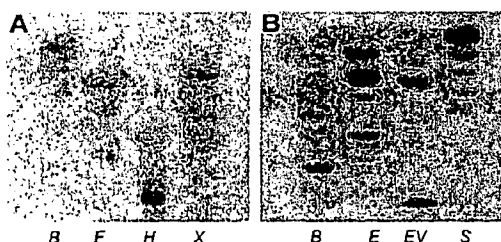


Figure 5. Genomic DNA Gel Blot Analysis of the *GhACT1* Gene.

Thirty micrograms of genomic DNA was digested with restriction enzymes as indicated and fractionated on a 0.8% agarose gel. DNA gel blots were hybridized with  $^{32}\text{P}$ -labeled *GhACT1* 5'-region gene-specific probe (0.8 kb) (A) and  $^{32}\text{P}$ -labeled *GhACT1* exon 3 probe (0.6 kb) (B). B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xba*I; EV, *Eco*RV; S, *Sac*I.

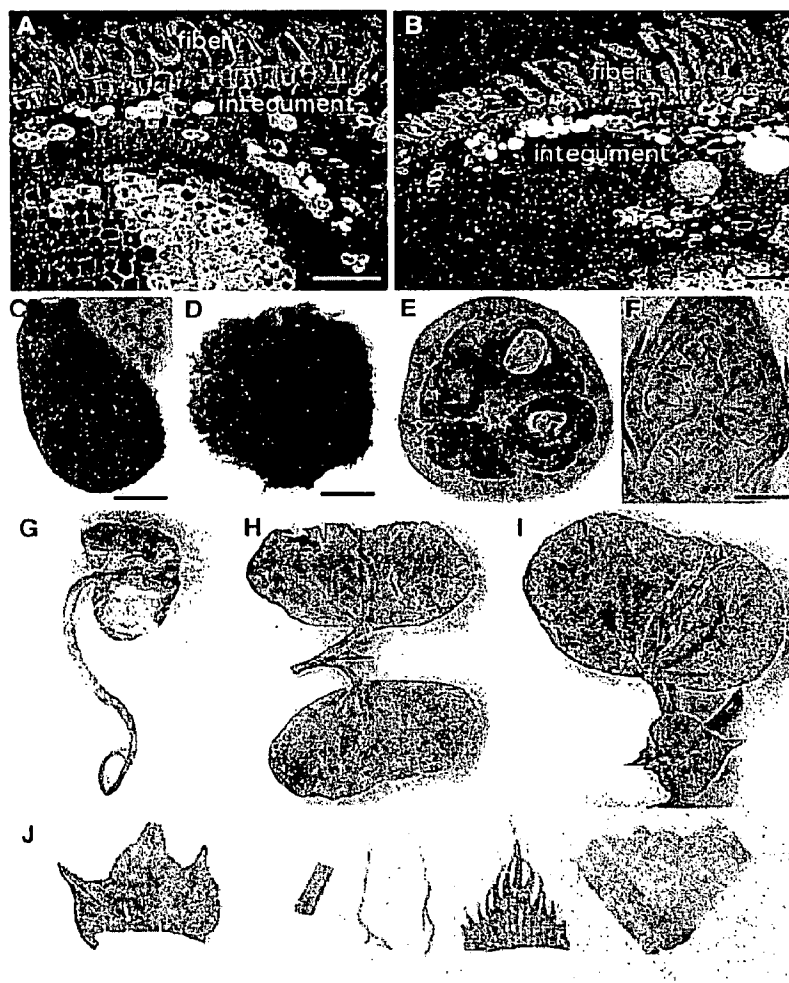
via *Agrobacterium tumefaciens*-mediated DNA transformation. A total of 230 transformed T0 plants from 21 independent calli were obtained and transplanted to soil for seeds. A total of 52 of 230 T0 transgenic plants were examined in detail for *GUS* expression patterns. In all of the 52 transgenic plants examined, strong *GUS* activity was detected only in young fibers (Figures 6A to 6E), whereas no or weak *GUS* staining was observed in ovules, anthers, petals, sepals, leaves, and roots, including their trichomes (Figures 6F and 6J). In comparison, plants transformed with the positive control pBI121 (35S::*GUS*) exhibited strong *GUS* activity in all tissues, and the nontransformed plants showed no *GUS* activity in fibers as well as in other tissues under the same staining conditions (data not shown). The same pattern of *GhACT1*::*GUS* expression was further confirmed in T1 and T2 transgenic plants. In addition, the *GhACT1*::*GUS* expression was observed at a moderate level in cotyledons of the germinating embryos at the first 1 to 2 d, when the root had just emerged from the embryo and the two cotyledons had not yet unfolded. In 3- to 4-d-old seedlings, moderate *GUS* activity was still observed in the cotyledon tissues (Figure 6G). Hypocotyls showed a low level of *GUS* activity in only one of the 21 independent transgenic lines examined. *GhACT1*::*GUS* expression was not detected in the roots of 3- to 8-d-old seedlings. Occasionally, weak expression was detected in the root tip in one transgenic line. As the seedling grew, *GUS* activity gradually decreased and finally disappeared in the cotyledons (Figures 6H and 6I). In 2-week-old seedlings, no significant *GUS* activity in the transgenic plants was detected. These results indicated that the 0.8-kb *GhACT1* promoter was sufficient to direct its fiber-specific expression and regulate its dynamic expression during cotton plant development.

#### Suppression of *GhACT1* Expression Dramatically Reduces Fiber Elongation

To study the role of actin cytoskeleton in fiber elongation, we chose a *GhACT1* gene that is expressed preferentially in fibers and less expressed in other tissues or organs (Figure 3A). Therefore, it was expected that knockdown of this gene would

have no or less effect on other tissues. Knockdown approaches using RNAi technology were employed. The 150-bp 3'-terminal fragment of *GhACT1* was constructed in the opposite direction with an intron from a cotton tubulin gene as a spacer (Li et al., 2002), then subcloned into pBI101 downstream of its own promoter (Figure 4C) and introduced into cotton cultivar Coker312 via *Agrobacterium*-mediated DNA transfer. Fourteen independent transgenic lines were regenerated. RNA gel blot analysis showed that the level of *GhACT1* mRNAs was reduced significantly down to very low level in fibers of the transgenic plants, using *GhACT1* 3'-UTR fragment as a probe (Figure 7A). To understand whether the reduced actin mRNAs also include other *GhACT* gene products, we further analyzed the expression levels of all the *GhACT* genes in fibers from RNAi transgenic plants by real-time quantitative SYBR-Green RT-PCR using gene-specific primers (Table 1). The results revealed that the expression of the *GhACT1* RNAi resulted in complete *GhACT1* silence in line T1 and ~10-fold reduction in lines T2, T3, and T4 (Figure 8). On the contrary, its impact on the expression of other *GhACT* genes was minor, with ~10% reduction (Figure 8). To confirm that the reduction in *GhACT1* mRNA also led to reduction at the actin protein level, protein gel blot analysis using actin antibody was performed. A strong band was detected in nontransgenic control fibers, whereas no or weak signals were detected in the transgenic lines (Figure 7B). This indicated that there was significant reduction in the actin proteins (mostly *GhACT1*) as a result of the reduction in *GhACT1* expression, and the remaining signals in the transgenic lines (Figure 7B, lanes 2 to 5) likely represented the other *GhACT* proteins expressed in fibers or residual *GhACT1*. These data suggest that *GhACT1* is one of the dominant and functional actin isoforms in fibers.

All *GhACT1* RNAi transgenic plants showed a short-fiber phenotype (Figure 9) that cosegregated with the kanamycin selection marker (data not shown) and the reduction of actin protein levels, indicating that the phenotype was a result of the actin reduction caused by *GhACT1* silence. Fiber cells differentiate and rapidly emerge from the surface of the ovule at 0 to 1 DPA in wild-type plants (Figure 9A), whereas fibers in transgenic plants (Figure 9D) were much shorter. At 2 DPA, fiber cells in wild-type plants reached ~500  $\mu\text{m}$  long (Figure 9B), whereas transgenic fibers were only ~150 to 380  $\mu\text{m}$  in length (Figure 9E). Fiber length at 3 DPA in most transgenic plants (Figure 9F) was equal to fibers at 2 DPA in wild-type cotton (Figure 9B) and much shorter than fibers at 3 DPA in wild-type plants (Figure 9C). Measurement of fiber length showed that fiber elongation in transgenic plants was ~1.5- to 3-fold slower than that in wild-type plants (Figure 10), which correlated with the reduction of actin protein level (see Figure 7B). The results suggest that the reduction in total actins, including *GhACT1*, slowed down fiber elongation. Moreover, a portion of the ovules was sterile, and bolls in transgenic plants were smaller than those in the wild type after maturation, indicating that *GhACT1* RNAi also slightly affected pollination or seed development. However, all the transgenic lines were unaffected in vegetative growth and flower development. No inhibition on fiber initiation was observed in the *GhACT1* transgenic lines, suggesting the *GhACT1* gene is most likely not involved in fiber initiation but plays a role in fiber elongation.



**Figure 6.** Histochemical Localization of *GUS* Activity in Transgenic Cotton Plants Containing the *GhACT1::GUS* Fusion Genes.

(A) and (B) Dark-field micrographs of 5- $\mu$ m-thick cross sections of 1- to 2-DPA ovules. A high level of *GUS* activity (represented by pink dots) was only found in the fiber, and very weak *GUS* staining was seen in the inner cell layers. No *GUS* staining was detected in the epidermal atrichoblast and integument.

(C) to (J) Bright field of micrographs or photographs of ovules and other tissues/organs.

(C) and (D) *GUS* staining in ovules at 1 (C) and 2 (D) DPA. Strong *GUS* activity was observed in the fibers.

(E) A cross section of a transgenic cotton boll at 14 DPA. Strong *GUS* activity was detected in the developing fibers, and very weak *GUS* staining was seen in embryos.

(F) A longitudinal section of a transgenic flower bud before anthesis. Weak *GUS* staining was found in some pollen grains.

(G) to (I) *GUS* staining in transgenic seedlings.

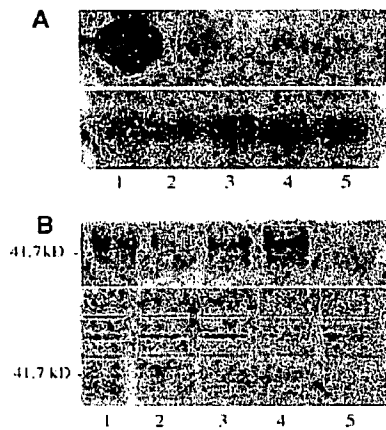
(G) Three-day-old seedling. *GUS* gene was expressed moderately in the cotyledons.

(H) Parts of a 7-d-old seedling. Weak *GUS* expression was found only in cotyledons.

(I) Parts of a 10-d-old seedling. *GUS* activity was very low in cotyledons, and no *GUS* expression was detected in other tissues, such as leaf and shoot apex.

(J) No *GUS* activity was detected in leaf, stem, root, sepal, and petal (from left to right) of transgenic cotton.

Bars = 80  $\mu$ m in (A) and (B), 1 mm in (C) and (D), and 2 mm in (F).



**Figure 7.** RNA Gel Blot and Protein Gel Blot Analyses of *GhACT1* Expression in RNAi Transgenic Fibers of Cotton.

(A) RNA gel blot analysis. Total RNAs from fibers at 10 DPA from a wild-type plant (1) and *GhACT1* RNAi transgenic lines (2 to 5) were fractionated on 1.2% denaturing agarose gel and transferred to a nylon membrane (see Methods). Top panel, autoradiograph of RNA gel blot hybridized with  $^{32}$ P-dCTP-labeled *GhACT1* probe; bottom panel, autoradiograph of the same RNA gel blot hybridized with  $^{32}$ P-dCTP-labeled 18S rRNA probe (control) showing equal loading of RNAs.

(B) Protein gel blot analysis. Total soluble proteins of fibers at 10 DPA from a wild-type plant (1) and *GhACT1* RNAi transgenic lines (2 to 5) were separated by electrophoresis in a 12% SDS-PAGE gel. The protein gel blot was stained with anti-actin antibody (top panel) and Coomassie blue (bottom panel).

#### Impact of *GhACT1* Suppression on the Actin Cytoskeleton in Fiber Cells

To investigate if changes in the actin cytoskeleton in *GhACT1* RNAi transgenic fibers occurred, we studied actin cytoskeleton in fiber cells using rhodamine-phalloidin staining for F-actin. During fiber cell elongation in wild-type plants, F-actin exhibited a complicated net-like structure from thin filaments to thick and longitudinally extending cables. At the early stage of fiber elongation, actin filaments were organized into arrays parallel to the growing axis and extended into the tip of the fiber cells (Figure 9G). With further elongation of fiber cells, the actin cytoskeleton was comprised of relatively thin arrays and thick cables along the long axis of the fiber (Figure 9H). The F-actin cytoskeleton displayed an increasingly complicated network consisting predominantly of thick and longitudinally long cables (Figure 9I). By contrast, actin filaments in transgenic fibers were obviously reduced in the number of filaments, and a more random array was observed (Figure 9J). During further development of the fibers, they were less bundled into arrays and cables (Figure 9K, compare with Figure 9H) and exhibited a defective F-actin organization (Figures 9K and 9L). As a result of RNAi of *GhACT1* expression in transgenic fibers, the devoid of the well-organized actin cytoskeleton consequently resulted in the reduction in fiber cell elongation, leading to the short-fiber phenotype. These data suggested that downregulation of

the *GhACT1* gene has an impact on actin cytoskeleton network in fiber cells.

## DISCUSSION

### Divergence of the Protein Structure of the GhACTs

Although plant actins are quite conserved, the divergence on protein structures occurred during evolution. In this study, the 16 cotton actins deduced from the isolated *GhACT* genes have diverged into nine subclasses compared with six subclasses in *Arabidopsis* (McDowell et al., 1996). Variation among *GhACTs* occurs more significantly than that found among the *Arabidopsis* actins. Figure 11 shows *GhACT1* protein structure, indicating that those significant substitutions on amino acids among the cotton actins may have an impact on their surface properties. The 14 positions where charged substitutions took place are found among the *GhACTs*, whereas only nine such positions appear among *Arabidopsis* actins (McDowell et al., 1996). At these positions, unlike *Arabidopsis* actins, only six positions were conservative substitutions, and the other positions showed nonconservative interchanges, whereas human actins contain only conservative substitutions. The nonconservative replacements of charged residues located on several surfaces of the actin molecule (Figure 11) may be involved in functional non-equivalency of actin isoforms as actin monomers polymerize from G-actin to F-actin and alter actin-actin or actin-actin binding protein interaction. For example, the uncharged polar Gln51 just adjacent to the DNase I binding loop involved in intermonomer interactions within the filament (Holmes et al., 1990) is replaced by a positively charged His in *GhACT5* and *GhACT6*. Because actin subdomain 2, in particular the DNase I binding loop, is directly involved in conformational changes (Otterbein et al., 2001), it is likely that this nonsynonymous substitution will have an impact on *GhACT5* and *GhACT6* structure and function. Recent genetic studies in *Arabidopsis* clearly showed that substitutions in *AtACT2* have dramatic impact on its functions in root hair development (Gilliland et al., 2002; Ringli et al., 2002; Diet et al., 2004). Missense mutation in *der1* mutants (Ala183Val in *der1-1*, Arg97His in *der1-2*, and Arg97Cys in *der1-3*) all caused deformed root hairs. Furthermore, Glu356Stop in *enl2* enhances *der1* phenotypically (Diet et al., 2004), indicating that C-terminal residues are not absolutely required for its function. On the contrary, the conservative substitutions in the N-terminal peptide of *Arabidopsis* actins may affect polymerization and myosin binding (McDowell et al., 1996). Either a charged (His or Arg) or an uncharged polar amino acid (Gln) at position 123 of cotton actins may suggest that Gln and His are functionally interchangeable. Besides the charged substitutions, there were four positions where a noncharged Gly was substituted by a charged Glu or Arg seen in *GhACT1* (Gly<sub>253</sub> to Glu), *GhACT6* (Gly<sub>184</sub> to Arg), *GhACT8* (Gly<sub>158</sub> to Arg), and *GhACT14* (Gly<sub>297</sub> to Glu), respectively. These substitutions are not found in *Arabidopsis* actin genes (McDowell et al., 1996). It would be interesting to know whether these nonsynonymous substitutions have structural and functional impacts on transition from G-actin to F-actin.

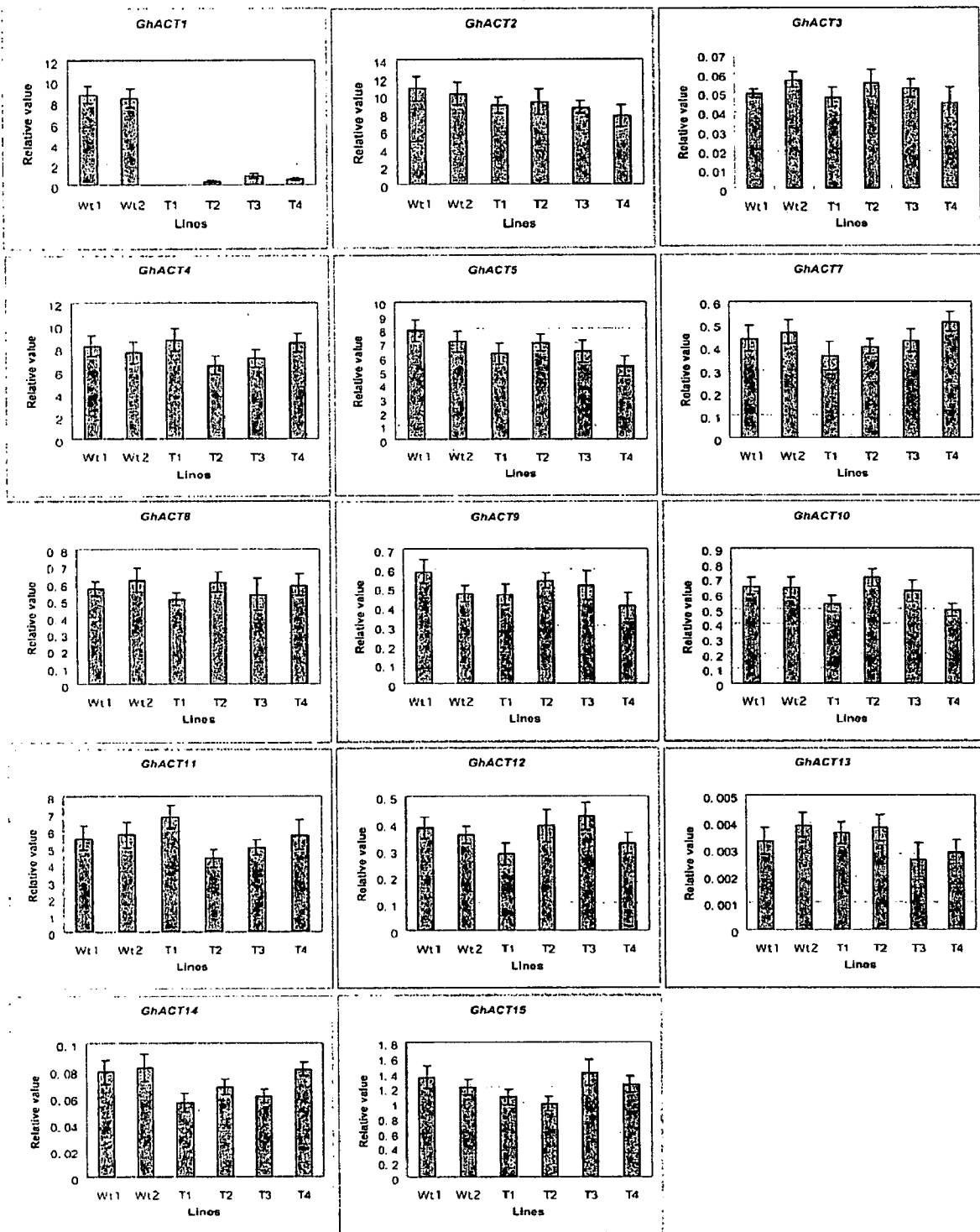


Figure 8. Real-Time RT-PCR Analysis of *GhACT1* RNAi Expression in Transgenic Fibers.

Relative value of *GhACT* gene expression in 8-DPA fibers is shown as a percentage of *GhUBI* expression activity (see Methods). The *GhACT1* expression was significantly silenced by RNAi in the transgenic fibers, whereas the activities of the other *GhACT* genes were little affected in fibers of all the transgenic lines. Wt1 and Wt2, wild-type plants; T1 to T4, transgenic *GhACT1* RNAi lines.



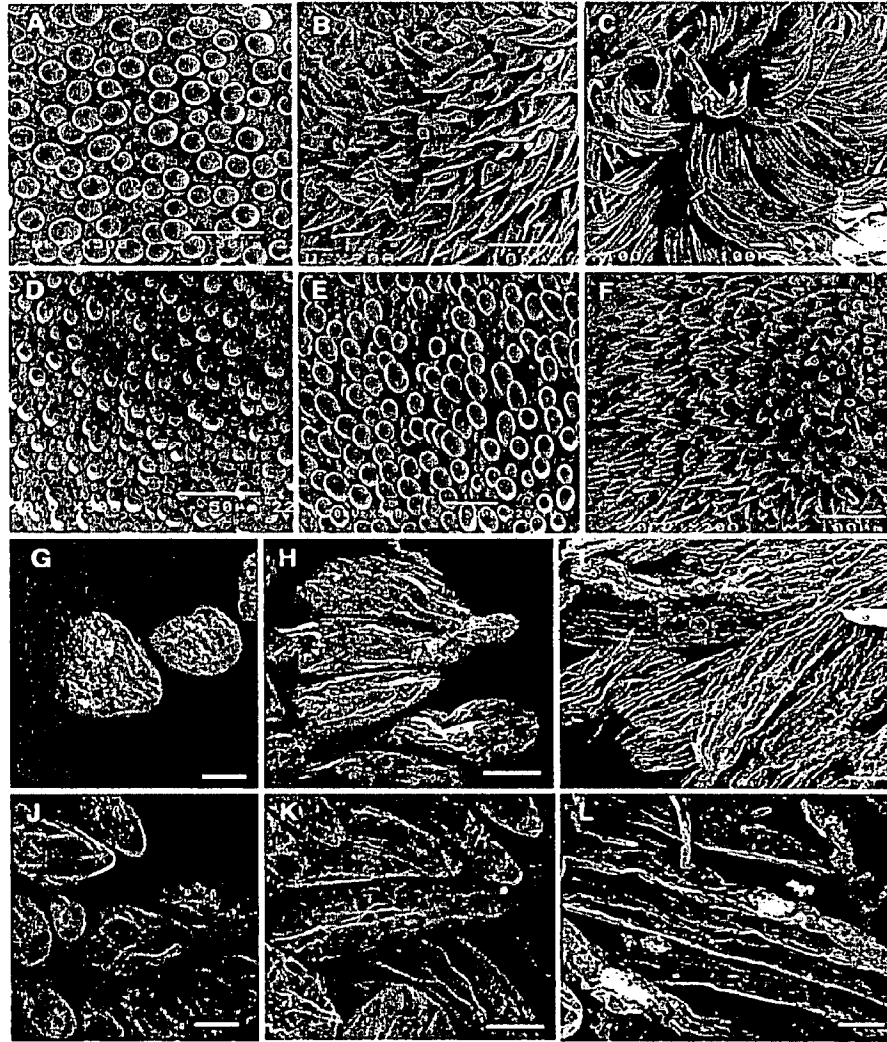


Figure 9. Comparison of Fiber Growth Rate and F-Actin Organization in Fiber Cells between Transgenic *GhACT1* RNAi and Wild-Type Plants.

(A) to (F) Scanning electron micrographs of the ovule surface of transgenic *GhACT1* RNAi and wild-type plants.

(A) to (C) Ovules of wild-type plants at 1 (A), 2 (B), and 3 (C) DPA. Note the length of fibers increases with time.

(D) to (F) Ovules of transgenic plants at 1 (D), 2 (E), and 3 (F) DPA. Note the length of fibers is much shorter than that in wild-type plants at the same stages.

(G) to (L) Organization of actin filaments in fiber cells of wild-type and transgenic *GhACT1* RNAi cotton.

(G) to (I) Fiber cells of wild-type cotton at 1 (G), 2 (H), and 3 (I) DPA. Actin filaments were organized into arrays parallel to the growing axis and extended into the tip of the fiber cells at 1 DPA (G). Actin filaments were arranged into thin arrays and thick cables along the shank in fiber cells at 2 DPA (H) and assumed a more complicated net structure of thick and longitudinally extending cables in >3-DPA fiber cells (I).

(J) to (L) Fiber cells of transgenic cotton at 1 (J), 2 (K), and 5 (L) DPA. Fewer F-actin cables were present.

Bars = 5  $\mu$ m in (G) and (J) and 10  $\mu$ m in (H), (I), (K), and (L).

A unique feature of the actin gene family is the position of introns that are conserved among actin genes in cotton and other plant species (Shah et al., 1983; Baird and Meagher, 1987; Nairn et al., 1988; Stranathan et al., 1989; McElroy et al., 1990; Meagher and Williamson, 1994; Cox et al., 1995; An et al.,

1996). In *Arabidopsis*, actin genes have three small introns at identical locations as in *GhACT* genes except *ACT2*, in which the first intron between codons 20 and 21 is missing (McDowell et al., 1996). In *GhACT1* and *GhACT2*, the first intron is rather large (545 and 565 bp, respectively) compared with the second and third

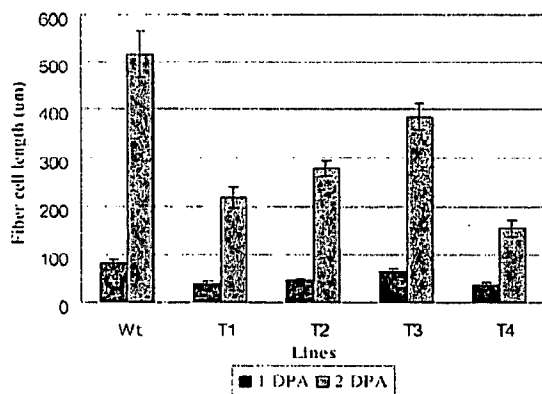


Figure 10. Fiber Length of Transgenic *GhACT1* RNAi and Wild-Type Cotton Seeds at 1 and 2 DPA.

Ovules were sectioned and the length of 30 fiber cells was measured under a microscope for each transgenic line and wild type. Data was processed with Microsoft Excel. Compared with the wild type, fiber cells of transgenic plants are much shorter and are approximately one-half to one-third of wild-type fibers.

introns (91 and 76 bp, respectively) that are more conserved, indicating that the first intron is more divergent than intron 2 and intron 3. Furthermore, unlike the other known plant actin genes studied, *GhACT1* and *GhACT2* share high similarity in the sequences of all three introns, suggesting that both genes may have very close evolutionary relationship.

Nevertheless, the triplet CAG insertion 4 bp upstream the second exon-intron junction in *GhACT8* challenged the conserved intron organization paradigm that the three introns are located at the same positions in all actin genes in the plant kingdom (McDowell et al., 1996). In actin genes examined so far, the second intron is always located at amino acid residue 152. However, in *GhACT8*, the second intron was located at amino acid residue 153 instead of residue 152 because of the insertion. The functional and evolutionary implications of this insertion remain unknown, although *GhACT8* is expressed at high levels in fiber and root.

#### *GhACT1* Is Preferentially Expressed during Fiber Development

In this study, we demonstrated that differential expression of the *GhACT* gene occurs in cotton, as did the members of this actin family in several other plant species, such as *Arabidopsis* (McDowell et al., 1996; Meagher et al., 1999b), soybean (McLean et al., 1990), tobacco (Thangavelu et al., 1993), and rice (McElroy et al., 1990). The differential expression data may imply that the specialized functional expressions of actin genes are required for proper development of the respective cell and tissue types and may reflect the divergent evolution of actin gene regulatory elements for expression in plant development.

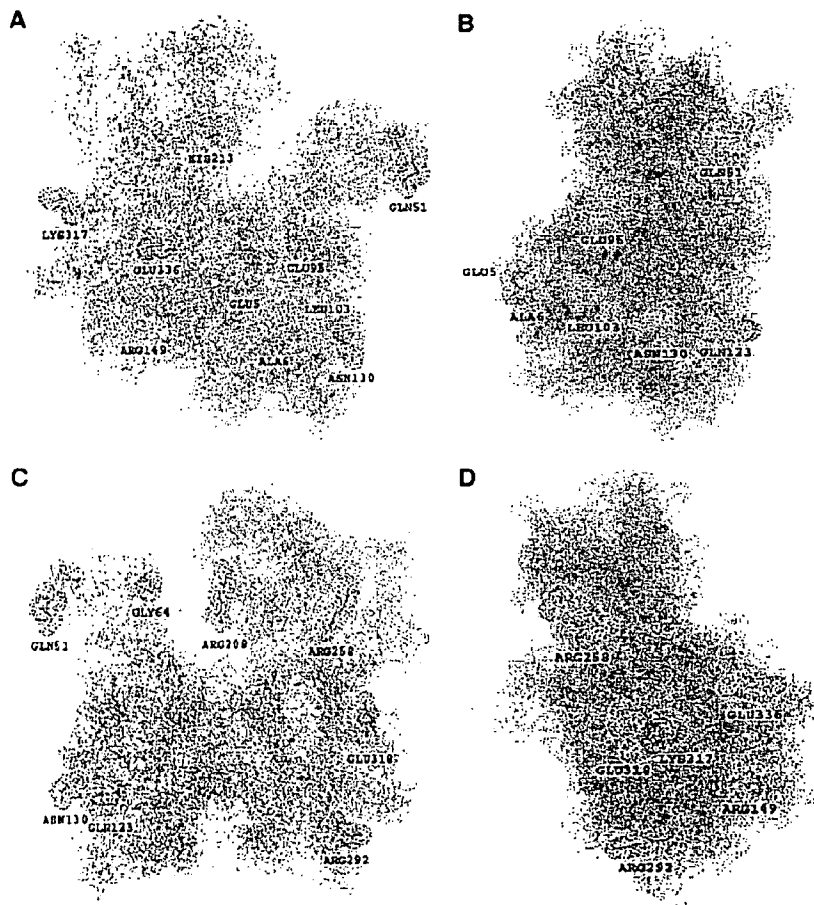
The data presented here provide evidence for strong expression of some *GhACT* genes in cotton fibers. The high level

of *GhACT* gene expression coincides with the rapid elongation of the fiber cell, suggesting that actin cytoskeleton plays an essential role in fiber elongation. It seems that specialized *GhACT* genes had been evolved to meet the requirement of the actin cytoskeleton for rapid fiber elongation. This is manifested by the fiber-specific expression of *GhACT1*, as well as *GhACT2* and *GhACT5*. Real-time RT-PCR and RNA gel blot analysis showed that the *GhACT1* transcripts accumulated preferentially in developing fibers, whereas only low or undetectable levels of RNAs were found elsewhere. The transcripts of *GhACT1* reach the highest level in young fibers during 8 to 14 DPA, and then there is a gradual and visible decrease of mRNA as the fiber cells developed further. Similarly, genes involved in osmoregulation and cell expansion during fiber development are also expressed at a high level (Orford and Timmis, 1998; Smart et al., 1998; Ruan et al., 2001). Consistently, *GhTua2/3* and *GhTua4* genes increased in abundance from 10 to 20 DPA, whereas *GhTua1* and *GhTua5* transcripts were abundant only through to 14 DPA and dropped significantly at 16 DPA with the onset of secondary wall synthesis (Whittaker and Triplett, 1999). Our previous study indicated that the *GhTUB1* gene was preferentially expressed in the early stage of fiber development (Li et al., 2002). This suggests that strict developmental control on genes, such as *GhACT1*, involved in cell elongation during cotton fiber and ovule development had evolved.

To study the developmental control mechanisms, we isolated the *GhACT1* gene and its promoter. The 0.8-kb 5' upstream sequence was cloned upstream the *GUS* reporter and transferred to cotton plants. *GUS* assay showed that the promoter is very active in developing fibers, whereas no or very little activity is present in leaf, stem, root, petal, and sepal. It should be emphasized that *GhACT1* was not expressed in leaf, stem, root, petal, and sepal trichomes, suggesting that the actin isotype encoded by *GhACT1* may be specific for fiber growth, rather than that of other trichomes. This is consistent with the *GhACT1* expression pattern revealed by real-time RT-PCR and RNA gel blot analysis, indicating that the 0.8-kb *GhACT1* promoter is sufficient to drive its tissue-specific expression and contains all the *cis* regulatory elements for its developmental regulation, as for actin genes in *Arabidopsis* (An et al., 1996; Huang et al., 1996; McDowell et al., 1996; Meagher et al., 1999b; Vitale et al., 2003). Thus, the 0.8-kb *GhACT1* promoter can be useful for isolating transcriptional factors that recognize the promoter sequence and for directing target gene expression in fiber cells. By comparing other fiber-specific promoter sequences such as *E6*, *H6*, and *FbL2A* (John and Crow, 1992; John and Keller, 1995, 1996; Rinehart et al., 1996), we hope to be able to identify fiber-specific *cis* elements and *trans* regulatory factors in the future.

#### *GhACT1* Plays a Major Role in Fiber Elongation

Fiber cell development, similar to trichome morphogenesis in leaf and stem (Mathur et al., 1999), requires the actin cytoskeleton for elaborating and maintaining the spatial patterning. During the early stage of fiber elongation, a rapid rate of actin turnover must keep pace with the equally rapid rates of fiber growth. The downregulation of *GhACT1* via RNAi technology in the transgenic fibers greatly reduces actin level that consequently affects



**Figure 11.** Significant Amino Acid Substitutions within Cotton GhACT Proteins.

The model was constructed using the spdbv37sp5 protein structure program (SwissModel first approach mode) from the Web site <http://swissmodel.expasy.org/> of the Swiss Institute of Bioinformatics. Front (A), right side (B), back (C), and left side (D) views, respectively, of the space-filling model for cotton GhACT1. The GhACT1 structure was built based on the known actin three-dimensional structure. Substitutions involving charged or strongly polar amino acid interchanges among the 15 cotton actin isoforms (GhACT1 to GhACT15) are shown in the labeled amino acid residues.

actin cytoskeleton organization, and as a result, fiber elongation is inhibited (Figure 9). This demonstrated that *GhACT1* plays a major role in fiber elongation, although we could not completely rule out the contribution of *GhACT* genes (such as *GhACT2* and *GhACT5*). The growth of cotton fiber cells is different from that of most other plant cells because of its rapid and synchronous tip elongation. It has been reported that F-actin plays an important role in pollen tube growth (Mascarenhas, 1993; Chen et al., 2002), in trichome morphogenesis (Mathur et al., 1999), in root hair tip growth (Miller et al., 1999), and in cell elongation of other cell types (Baluska et al., 2000; Waller et al., 2002; Yamamoto and Kiss, 2002). In the tip-growing pollen tube, F-actin arrays are very dynamic, changing from large spherical bodies to F-actin bundles oriented predominantly parallel to the growth axis (Tiwari and Polito, 1988). Similar F-actin arrays were also found

for root hair growth (Miller et al., 1999) as well as root tip growth (Blancaflor and Hasenstein, 1997). However, *Arabidopsis act7* mutants showed remarkably reduced F-actin in the cells of the root elongation zone. As a result, mutants displayed a series of abnormal phenotypes, such as delayed and less efficient germination, increased root twisting and waving, and retarded and slowed root growth (Gilliland et al., 2003). The *act2-1* insertion fully disrupted *ACT2* gene expression and significantly decreased the level of total actin protein, resulting in much shorter root hairs (Gilliland et al., 2002; Ringli et al., 2002). Immunocytochemical analysis revealed only several thin actin bundles in the short mutant root hairs, and in the very apex of these stunted root hairs, the actin bundles were often looping through the tip or showing dense but diffuse fluorescence labeling (Gilliland et al., 2002). It was believed that actin isovariants of *Arabidopsis* have

evolved distinct reproductive and vegetative functions and have showed functional nonequivalency among each other. Mis-expression of the pollen-specific reproductive ACT1 isovariant in vegetative tissues altered actin polymerization and F-actin organization and thereby dramatically affects plant development and morphogenesis (Kandasamy et al., 2002). In cotton fiber, we found that the F-actin was organized into short and thin arrays in tip orientation at the early stage of fiber development, and with further development, F-actin cytoskeleton displayed an increasingly complicated net-like structure consisting predominantly of thick and longitudinally extending cables in fiber cells. On the other hand, when GhACT1 isovariant level was reduced significantly in the transgenic fiber cells, F-actin bundles were reduced with only a few filaments (Figure 9), similar to *act7* and *act2* mutants. The reduced and defective actin cytoskeleton was unable to meet rapid fiber elongation. This suggested that F-actin arrays maintained by a significant amount of actins (mostly GhACT1) is critical for fiber cell elongation, like in root hair and trichome cell types.

F-actin might transport vesicles toward the cell periphery, especially near the polar region during tip growth. When the movement of F-actins was blocked, the vesicles could not be released in the cell periphery (polar region), resulting in the inhibition of the coleoptile cell elongation (Waller and Nick, 1997; Waller et al., 2002). In root hair development of *Vicia sativa*, the elongating net-axial fine bundles of actin filaments (FB-actin) function in polar growth by targeting and releasing Golgi vesicles to the vesicle-rich region of the hair cells. When the elongation of FB-actin was blocked by cytochalasin D, the tip growth of root hair cells was stopped (Miller et al., 1999). In our study, because of suppression of *GhACT1* expression, the reduction of F-actin level in transgenic fibers might have a similar effect on fiber cells. With the reduction of actin filaments, the number of organelles (such as Golgi body and endoplasmic reticulum) traveling along the filaments may decrease significantly in the *GhACT1* RNAi transgenic fiber cells. The significant reduction in vesicles may account for the slow elongation of fiber cells in the transgenic plants.

In conclusion, our results provide direct evidence that *GhACT1*, perhaps as well as other *GhACT* genes (such as *GhACT2* and *GhACT5*), is involved in fiber elongation, not fiber initiation. The characterization and expression studies give us novel insights into the role of *GhACT1* in cotton fiber development. Furthermore, the *GhACT1* promoter provides a useful tool to identify transcription regulators confirming its fiber-specific expression and to direct potential target genes for fiber quality improvement.

## METHODS

### Plant Materials

Cotton (*Gossypium hirsutum* cv Coker312) seeds were surface-sterilized with 70% ethanol for 30 to 60 s and 10% H<sub>2</sub>O<sub>2</sub> for 30 to 60 min, followed by washing with sterile water. The sterilized seeds were germinated on half-strength MS medium under a 12-h-light/12-h-dark cycle at 28°C. Cotyledons and hypocotyls were cut from sterile seedlings as explants for transformation as described before (Li et al., 2002). Tissues for DNA and

RNA extraction were derived from cotton plants (*G. hirsutum* cv DP5415 and Xuzhou142) grown in a greenhouse.

### Construction of Cotton cDNA Libraries

Total RNA was extracted from young fibers, ovules, anthers, petals, leaves, cotyledons, and roots as described previously (Li et al., 2002). Poly(A)<sup>+</sup> mRNA was purified from total RNA using an mRNA purification kit (Qiagen, Düsseldorf, Germany). cDNA was synthesized and cloned into the *EcoRI*-*XhoI* sites of the ZAP Express vector and packaged using a ZAP-cDNA Gigapack Gold III cloning kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

### Isolation of *GhACT* cDNAs and RNA Gel Blot Analysis

More than 300 cDNA clones were randomly selected from the cotton fiber cDNA library for sequencing. Sequence analysis identified one actin clone, *GhACT1*. The 380-bp fragment of the 3'-UTR of *GhACT1* was obtained by PCR amplification using primers *GhACT1*-3'L (5'-AGTTTGTAAATGCTTTTGATGGT-3') immediately downstream the stop codon and *GhACT1*-3'R (5'-AAATCTCGTACAATAATAGCTATT-3') and used as a gene-specific probe for RNA gel blot analysis as described previously (Li et al., 2002). Then, a 600-bp fragment representing *GhACT1* exon 3 was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP and used as a probe to screen a cotton cDNA library according to standard procedures (Sambrook et al., 1989). cDNA (5 × 10<sup>6</sup>) clones were screened, and 300 clones were identified. Among them, 60 full-length clones were sequenced and analyzed. In total, 15 unique cDNA clones were obtained.

### Sequence and Phylogenetic Analysis

Nucleotide and amino acid sequences were analyzed using DNASTar (DNASTar, Madison, WI). For phylogenetic analysis, 15 *GhACT* peptide sequences and one putative cotton actin sequence (AF059484) were aligned with the ClustalW program (<http://www.ebi.ac.uk>), then maximum parsimony analysis was performed with the PAUP 4.0 program (Swofford, 1998) using yeast actin ScACT1 as an outgroup. The heuristic search methods were applied and the best parsimonious trees were retained in each search.

### RT-PCR Analysis

The expression of the *GhACT* genes in cotton tissues was analyzed by real-time quantitative RT-PCR using the fluorescent intercalating dye SYBR-Green in a LightCycler detection system (Roche, Indianapolis, IN). A cotton polyubiquitin gene (*GhUB1*) was used as a standard control in the RT-PCR reactions. A two-step RT-PCR procedure was performed in all experiments. First, total RNA samples (2  $\mu$ g per reaction) from leaves, stems, cotyledons, roots, anthers, petals, and fibers were reversely transcribed into cDNAs by AMV reverse transcriptase according to the manufacturer's instructions (Roche). Then, the cDNAs were used as templates in real-time PCR reactions with gene-specific primers (Table 1). The real-time PCR reaction was performed using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. The amplification of the target genes was monitored every cycle by SYBR-Green fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure for the starting copy numbers of the target gene. Relative quantitation of the target *GhACT* expression level was performed using the comparative Ct method (Roche LightCycler system). The relative value for expression level of each *GhACT* gene was calculated by the equation  $Y = 10^{\Delta Ct/3} \times 100\%$  ( $\Delta Ct$  is the differences of Ct between the control *GhUB1* products and the target

GhACT products; i.e.,  $\Delta Ct = Ct_{GhUBI} - Ct_{GhACT1}$ ). To achieve optimal amplification, PCR conditions for every primer combination were optimized for annealing temperature and  $Mg^{2+}$  concentration as recommended by the Roche LightCycler system instructions. PCR products were confirmed on an agarose gel. The efficiency of each primer pair was detected using GhACT cDNAs as standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair.

#### DNA Gel Blot Analysis

Genomic DNA was isolated from young cotton (*G. hirsutum* cv DP5415 and Xuzhou142) leaves using a modified method described earlier (Li et al., 2002). Genomic DNA was digested with restriction enzymes and separated on 0.7% agarose gels and transferred onto Hybond N<sup>+</sup> nylon membranes (Amersham Biosciences, Buckinghamshire, UK) by capillary blotting. DNA gel blot hybridization was performed at 68°C overnight using ExpressHyb solution (Clontech, Palo Alto, CA) with <sup>32</sup>P-labeled gene-specific DNA probes prepared by the Prime-a-Gene labeling system (Promega, Madison, WI), followed by washing at 68°C in 0.1× SSC and 0.5% SDS for 30 to 60 min. The <sup>32</sup>P-labeled membranes were exposed to x-ray film at -80°C for 1 to 3 d.

#### Isolation of GhACT Genes by Screening Cotton Genomic Libraries

Cotton genomic libraries were constructed as described earlier (Li et al., 2002). Approximately  $2 \times 10^6$  clones were screened with a [ $\alpha$ -<sup>32</sup>P]dCTP-labeled GhACT1 (0.6 kb of exon 3) probe generated by the Prime-a-Gene labeling system (Promega). The membranes (Hybond N<sup>+</sup>; Amersham Biosciences) were hybridized overnight in ExpressHyb solution (Clontech) at 68°C, followed by washing with 0.1× SSC and 0.5% SDS. Autoradiography was performed with x-ray film (Kodak, Rochester, NY), and positive clones were purified and sequenced with the ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

#### Construction of the GhACT1::GUS Chimeric Gene and GhACT1 RNAi

Primers at -816 to -793 bp with an introduced *SaI* site and from -1 to -27 bp before ATG with an introduced *XbaI* site were used to amplify the GhACT1 promoter. A 0.8-kb PCR fragment was obtained using pfu DNA polymerase (Stratagene, La Jolla, CA) and digested with *SaI* and *XbaI*, then subcloned into the *SaI*/*XbaI* sites of the pBI101 vector (Clontech) to generate a chimeric GhACT1::GUS gene (named pBI-ACT1-p) (Figure 4B).

To construct GhACT1 RNAi vector, the first intron (0.2 kb) of the *GhTUB1* gene (Li et al., 2002) was amplified by PCR with two introduced sites, *XbaI* and *SpeI*, using the primer pair TUBint-L, 5'-GGGTCTAGAGACGTAGTTAGAAAGGAAGCCGA-3', and TUBint-R, 5'-GGGACTAGTACGTTCCCATTCGGAACCCGTT-3', and inserted into a pBluescript II SK+ vector at the sites *XbaI* and *SpeI* to obtain an intron-containing intermediate construct (pSK-TUBint). The GhACT1 3'-terminal sequence (150 bp fragment at 229 to 378 bp downstream the stop codon) was cloned into the 5' arm with the introduced sites *Bam*HI/*SpeI* and the 3' arm with the introduced sites *XbaI*/*SacI* of the intron in pSK-TUBint vector for the sequences encoding the inverted repeat RNA. The constructed RNAi of the GhACT1 gene was subcloned into the GhACT1::GUS construct at *Bam*HI/*SacI* sites to replace the GUS gene (named pBI-TUBint-ACT1i) (Figure 4C).

#### Cotton Transformation

Cotyledon and hypocotyl explants from *G. hirsutum* cv Coker 312 were transformed using *Agrobacterium tumefaciens*-mediated transformation as described previously (Li et al., 2002). Homozygosity of transgenic

plants was determined by segregation ratio of the kanamycin selection marker and further confirmed by DNA gel blot analysis.

#### Histochemical Assay of GUS Gene Expression

Histochemical assays for GUS activity in transgenic cotton plants were conducted according to Jefferson et al. (1987), with slight modification. Fresh plant tissues were incubated in 5-bromo-4-chloro-3-indolylglucuronide solution at 37°C for 4 to 8 h and then cleared and fixed by rinsing with 100 and 70% ethanol successively. For sectioning, 1 to 3 DPA ovules stained in 5-bromo-4-chloro-3-indolylglucuronide solution were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, overnight at room temperature, then dehydrated through conventional ethanol series, and finally embedded in Historesin (Leica, Wetzlar, Germany) according to the manufacturer's instructions. The samples were cut into 5- to 7- $\mu$ m-thick sections using a Leica microtome. The sections were examined and photographed under a Leica DMR microscope equipped with dark-field optics.

#### Protein Gel Blot Analysis

Soluble proteins were extracted from 8 to 10 DPA wild-type and GhACT1 RNAi transgenic fibers in extraction buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 mM  $CaCl_2$ ,  $\alpha$ -mercaptoethanol, 0.5% Nonidet P-40, 1  $\mu$ g/mL aprotinin, 1  $\mu$ g/mL leupeptin, and 0.6  $\mu$ L/mL PMSF. Protein concentration was determined by the Bradford method. Equal amounts of proteins were separated by electrophoresis in a 12% SDS-PAGE gel and transferred onto a nylon membrane by electric transfer (Trans-Blot system; Bio-Rad, Hercules, CA) using semidry transfer buffer. The membrane was blocked with 5% nonfat milk in PBS buffer containing 0.05% Tween-20 at room temperature for at least 1 h and then incubated with affinity-purified goat polyclonal anti-actin IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After washing in PBS buffer for 30 min, the membrane was incubated in PBS containing horseradish peroxidase-conjugated rabbit anti-goat IgG (Pierce, Rockford, IL) for 1 h. After washing in PBS buffer, the membrane was incubated in SuperSignal West Substrate (Pierce) working solution for 5 min and then exposed to x-ray film.

#### Scanning Electron Microscopy

For examining fiber initiation and elongation, fresh ovules were dissected out and placed on double-sided sticky tape on an aluminum specimen holder and frozen immediately in liquid nitrogen. The frozen sample was viewed with a JSM-5310LV scanning electron microscope (JEOL, Tokyo, Japan).

#### Observation of F-Actin Structures in Fiber Cells

Ovules dissected out from fresh bolls at 1 to 4 DPA, with or without maleimidoenzoyl-*N*-hydroxysuccinimide ester pretreatment, were fixed in a solution of 2% paraformaldehyde in KMCP buffer (70 mM KCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , and 100 mM Pipes, pH 6.5) for 1 h. After rinsing in KMCP buffer, the ovules were sectioned into slices of ~1 mm thickness. Thin sections were transferred to slides and treated with 1% cellulase (Sigma-Aldrich, St. Louis, MO), 0.5% hemicellulase (Sigma-Aldrich), 0.5% pectinase (Sigma-Aldrich), and 0.1% BSA in KMCP buffer for 10 min, followed by washing with KMCP buffer. Finally, sections were incubated in a solution of 5  $\mu$ g/mL Phalloidin-TRITC (Sigma-Aldrich) in KMCP buffer with 0.1% Triton X-100 at room temperature. Excess phalloidin was removed by rinsing with the same buffer. Stained ovule sections were immediately examined with an LSM510 confocal microscope (Zeiss, Jena, Germany).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY305723 to AY305737.

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# Molecular analysis of the stylar-expressed *Solanum chacoense* small asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*

M. O'Brien<sup>1</sup>, C. Kapfer<sup>1</sup>, G. Major<sup>1</sup>, M. Laurin<sup>1</sup>, C. Bertrand<sup>1</sup>, K. Kondo<sup>2</sup>, Y. Kowiyama<sup>2</sup> and D. P. Matton<sup>1,\*</sup>

<sup>1</sup>Département de Sciences Biologiques, Institut de recherche en biologie végétale, Université de Montréal, 4101 Sherbrooke est, Montréal, Québec, Canada H1X 2B2, and

<sup>2</sup>Faculty of Bioresources, Mie University, Tsu 514-8507, Japan

Received 12 July 2002; revised 20 August 2002; accepted 16 September 2002.

\*For correspondence (fax +1 514 872 9406; e-mail mattond@irbv.umontreal.ca).

## Summary

Gametophytic self-incompatibility (GSI) systems involving the expression of stylar ribonucleases have been described and extensively studied in many plant families including the Solanaceae, Rosaceae and Scrophulariaceae. Pollen recognition and rejection is governed in the style by specific ribonucleases called S-RNases, but in many self-incompatibility (SI) systems, modifier loci that can modulate the SI response have been described at the genetic level. Here, we present at the molecular level, the isolation and characterization of two *Solanum chacoense* homologues of the *Nicotiana* HT modifier that had been previously shown to be necessary for the SI reaction to occur in *N. alata* (McClure *et al.*, 1999). HT homologues from other solanaceous species have also been isolated and a phylogenetic analysis reveals that the HT genes fall into two groups. In *S. chacoense*, these small proteins named ScHT-A and ScHT-B are expressed in the style and are developmentally regulated during anthesis identically to the S-RNases as well as following compatible and incompatible pollination. To elucidate the precise role of each HT isoform, antisense ScHT-A and RNAi ScHT-B lines were generated. Conversion from SI to self-compatibility (SC) was only observed in RNAi ScHT-B lines with reduced levels of ScHT-B mRNA. These results confirm the role of the HT modifier in solanaceous SI and indicate that only the HT-B isoform is directly involved in SI.

**Keywords:** self-incompatibility, S-RNase, Solanaceae, HT-modifier gene, RNA interference.

## Introduction

Self-incompatibility (SI) constitutes an important mechanism for preventing inbreeding through specific pollen recognition and rejection. In the most widespread type of gametophytic self-incompatibility (GSI), the haploid pollen is rejected when the S-allele it expresses, matches either of the two S-alleles expressed in the sporophytic tissue of the pistil. For *Solanaceae*, the GSI phenotype is specified by a highly multiallelic S-locus (de Nettancourt, 1977, 1997) whose only known product is a secreted ribonuclease (McClure *et al.*, 1989) expressed in the transmitting tissue of the style (Anderson *et al.*, 1986; Matton *et al.*, 1998) and called an S-RNase. Gain-of-function experiments in SI plants have shown that expression of an S-RNase transgene is sufficient to alter the SI phenotype of the pistil but

not that of pollen (Lee *et al.*, 1994; Matton *et al.*, 1997; Murfett *et al.*, 1994). Furthermore, transgenic plants made to express high levels of S-RNase in pollen did not acquire the new phenotype (Dodds *et al.*, 1999), indicating that the pollen S gene (unknown to date) is clearly distinct from the S-RNase (Kao and McCubbin, 1996). In order to determine if expression of an active S-RNase is the sole determinant of SI in styles, transformation of closely related self-compatible (SC) species with S-RNases were attempted. Transformation of SC *Nicotiana tabacum* or *N. plumbaginifolia* with an S-allele from the SI species *N. alata* did not result in the acquisition of the SI phenotype (Murfett *et al.*, 1996), nor did the introgression of a chromosome fragment bearing the S-locus from the SI *Lycopersicon hirsutum* in SC



*L. esculentum* (Bernatzky *et al.*, 1995), or the expression of an S-RNase from the SI *L. peruvianum* in the SC *L. esculentum* (Kondo *et al.*, 2002b). Conversely, when an S-allele from the SC *Petunia hybrida* was introduced in SI *P. inflata*, it became functional in rejecting its corresponding self-pollen, indicating that factors expressed in the *P. inflata* SI genetic background were needed for the SI reaction to occur (Ai *et al.*, 1991). These results strongly suggest that other factors are necessary for the SI reaction to occur. Some of these factors that affect the SI response have been described in numerous SI systems and often been named S-locus inhibitors or modifiers (de Nettancourt, 1977). In *S. chacoense*, an S-locus inhibitor (Sli) has been mapped to the distal end of chromosome 12, but has not been cloned yet (Hosaka and Hanneman, 1998a,b). To date, the only modifier functionally characterized at the molecular level is the HT gene, a stylar-expressed small asparagine-rich protein in *N. alata* (McClure *et al.*, 1999). The NaHT (*Nicotiana alata* HT) cDNA was isolated from a differential screen for SI stylar-specific transcripts, and antisense *Nicotiana* HT plants became SC, although they still expressed normal levels of stylar S-RNases. Recent correlative evidences from mRNA expression studies in *Lycopersicon* species also suggest the involvement of the HT modifier in SI (Kondo *et al.*, 2002a,b). Here, we describe the characterization of HT homologues that are co-ordinately expressed with the S-RNases during pistil development in the SI species *S. chacoense*, and show that only the HT-B isoform is involved in SI.

## Results

### Isolation of the *Solanum* HT homologues and sequence comparison

The ScHT-A<sub>1</sub>, ScHT-A<sub>2</sub> and S<sub>14</sub>-RNase cDNAs were isolated from a pollinated pistil cDNA library (see Experimental procedures section). The ScHT-A<sub>1</sub> cDNA codes for a small protein of 99 amino acid residues with a highly predicted N-terminal signal peptide as determined from the SignalP algorithm (Nielsen *et al.*, 1997). The predicted cleavage site for ScHT-A<sub>1</sub> is before Arg-25, producing a mature polypeptide of 75 amino acids (8 kDa). The ScHT-A<sub>2</sub> cDNA is incomplete in the 5' region, but would comprise all of the mature protein (77 residues, 8.3 kDa) as predicted from the ScHT-A<sub>1</sub>-deduced cleavage site. Both ScHT-A<sub>1</sub> and ScHT-A<sub>2</sub> predicted mature proteins are acidic with pIs of 3.98 and 4.11, respectively. Amino acid sequence comparison of the predicted mature polypeptides indicate that ScHT-A<sub>1</sub> and ScHT-A<sub>2</sub> are 96% identical (93% nucleotide sequence identity) and most probably correspond to allelic variants of the same gene (see linkage analysis of the ScHT-A isoforms below). The ScHT-B<sub>1</sub> isoform was obtained by PCR ampli-

fication with an upstream primer located in the signal peptide region and a downstream primer located 3' of the predicted stop codon from the *N. alata* HT and *S. chacoense* HT-A<sub>1</sub> isoforms. The ScHT-B<sub>1</sub> mature protein comprises 79 amino acids (MW, 8.7 kDa) with an acidic pI of 4.67, and is approximately 51% identical (57% similar) at the amino acid level to the ScHT-A isoforms. No N-glycosylation sites are found on either polypeptides, but six cysteine residues that could be involved in disulfide bonding are conserved between all HT homologues, except from the *S. pinnatisectum* B<sub>1</sub> isoform that lacks one cysteine, and are found flanking a striking C-terminal region containing 16–20 Asp (D) or Asn (N) residues. In the mature ScHT proteins, asparagine and aspartic acid residues account for roughly 30% of the total amino acids. A sequence alignment of the deduced amino acid sequences corresponding to the mature protein region of the *S. chacoense* HT isoforms as well as HT homologues from other SI solanaceous plants, including *L. peruvianum*, *N. alata*, *S. pinnatisectum*, *S. bulbocastanum* and from the SI species *S. tuberosum*, is shown in Figure 1(a). All *Solanum* and *Lycopersicon* sequences were obtained by PCR amplification with the same primer pairs as described for the amplification of ScHT-B<sub>1</sub>. Although all the HT sequences share some specific structural features, e.g. a C-terminal Asn/Asp-rich region flanked by conserved cysteine residues, they can be easily classified in two groups when the amino-terminal half of the protein is considered. Based on the CLUSTALX alignment, a phylogenetic analysis was performed to determine if this preliminary classification would hold true. Figure 1(b) shows that all the B isoforms fell into a highly supported cluster, while more sequence data would be needed to determine if the A-type sequences form one or more group. Interspecific amino acid sequence identities between the predicted mature polypeptides ranges from 76 to 86% in the A-isoform group, and 36–92% in the B-isoform group. The ScHT-A<sub>1</sub> and ScHT-A<sub>2</sub> (94%), SbHT-B<sub>1</sub> and SbHT-B<sub>2</sub> (98%) and SpHT-B<sub>1</sub> and SpHT-B<sub>2</sub> (97%) are most probably alleles of the same genes in their respective species. When the only non-*Solanum* sequence is removed (NaHT-B), the B-isoform group sequence identity is in the range of 77–92%. One surprising feature is the very high conservation of the predicted signal peptides between species, as determined from the available complete HT cDNA sequences (ScHT-A<sub>1</sub>, NaHT-B, LpHT-A<sub>1</sub> and LpHT-B<sub>1</sub>), ranging from 66 to 100% identity (82–100% similarity), when compared to the mature protein sequences (data not shown). This intriguing situation is also observed with the sporophytic SI (SSI) pollen S gene where the signal peptides are also far more similar to each other (mean of 77% identity and 89% similarity) than the mature protein sequences (29% identity and 38% similarity on average) when the sequences of five different SSI pollen S genes are compared (Schopfer *et al.*, 1999; Takayama *et al.*, 2000).

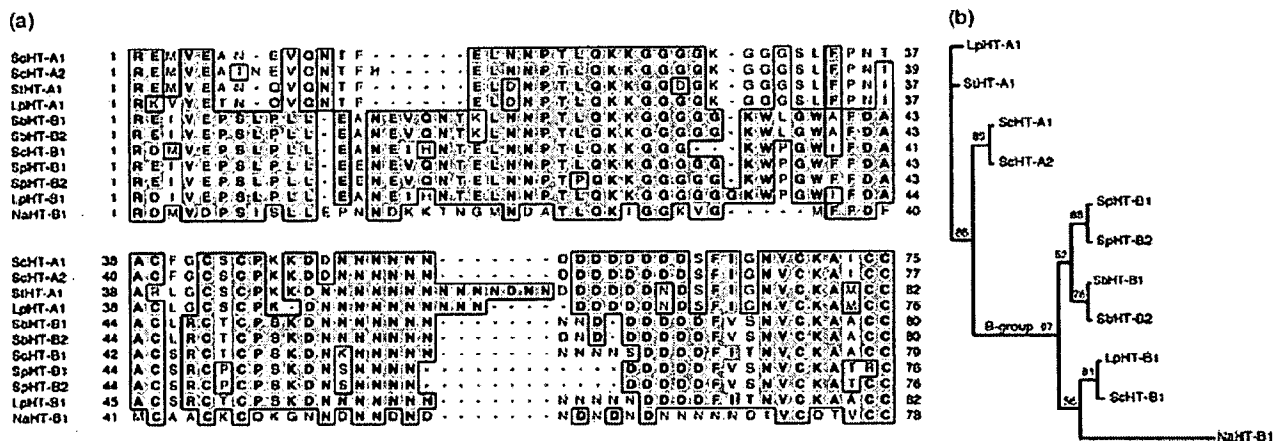


Figure 1. Sequence alignment (a) and phylogenetic analysis (b) of the deduced mature protein sequences of *ScHT-A<sub>1</sub>*, *ScHT-A<sub>2</sub>* and *ScHT-B<sub>1</sub>* with related sequences from other solanaceous species.

(a) CLUSTALX alignment was used to produce a phylogenetic analysis of related HT sequences in six solanaceous species.

(b) A jackknife analysis using Paup 4.08b was used to produce the phylogram which is shown.

#### Tissue-specific and developmental regulation of the *ScHT* modifiers

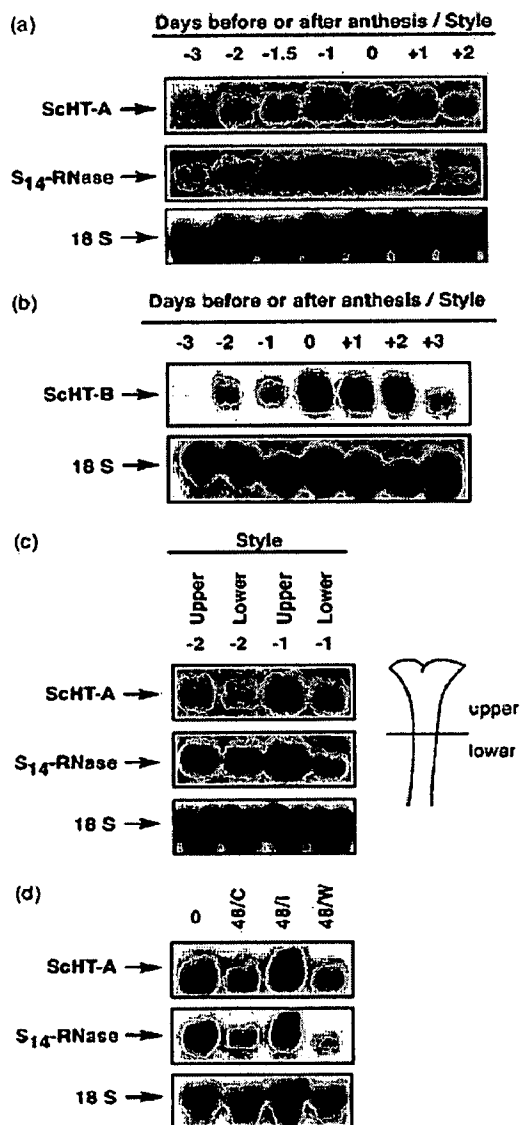
Tissue-specific expression of *ScHT-A* and *ScHT-B* isoforms was determined using RNA extracted from different tissues of *S. chacoense*. Since the *ScHT-A<sub>1</sub>* and *ScHT-A<sub>2</sub>* cDNAs are 93% identical at the DNA level, the RNA-gel blot analyses most probably reflect the expression of both genes, although the probe used at all time was *ScHT-A<sub>1</sub>*. Overall DNA sequence identity between the *ScHT-A* and *ScHT-B* isoforms is around 73%, and long stretches of identity might also produce cross-hybridization. In order to avoid this, an oligonucleotide specific to the B isoform and corresponding to the N-terminal sequence, PSLPLLEA, was synthesized. Both *ScHT-A* and *ScHT-B* isoforms are almost exclusively expressed in styles with very weak expression detected in ovary upon prolonged exposures (data not shown). No *ScHT-A* or *B* mRNAs could be detected in leaf, stem, root, petal, anther, pollen or pollen tube tissues (data not shown). This expression pattern is identical to the one observed for the *S-RNases* (Matton *et al.*, 1998). Since the *S-RNase* genes are themselves developmentally regulated during anthesis (Anderson *et al.*, 1986; Cornish *et al.*, 1987), we determined the RNA expression pattern of *ScHT-A* and *ScHT-B*, and compared with the one obtained from *S<sub>14</sub>-RNase* (Figure 2a,b). Both *ScHT* isoforms and the *S<sub>14</sub>-RNase* are identically regulated during pistil development and reach a maximum level of expression around anthesis day (Figure 2a,b). Figure 2(a,b) also shows that, in unpollinated flowers, *ScHT-A*, *ScHT-B* and *S<sub>14</sub>-RNase* mRNA levels decline from around 2 days after anthesis, coinciding with a reduced fertilization receptivity.

In *S-RNase*-mediated GSI, rejection of the pollen tubes mostly occurs in the top half of the style. To determine if

there could be a correlation with pollen tube arrest and the expression levels of genes involved in SI, mRNA levels of *ScHT-A* and *S<sub>14</sub>-RNase* were measured in the upper and lower halves of styles around peak expression time (Figure 2c). Both genes were more strongly expressed in the upper half of the style, consistent with the site of most pollen tube arrest as determined by aniline blue staining in *S. chacoense* styles (Matton *et al.*, 1999).

#### Effect of compatible and incompatible pollination on *ScHT* and *S-RNase* gene expression

In many species, pollination is known to induce deterioration and death of the secretory cells in the stigmatic region and in the transmitting tissue of the style (Cheung, 1996). We have previously shown that some genes that respond to pollination, also respond to wounding stress and wound hormone treatments, mainly jasmonates (Lantin *et al.*, 1999a,b). Wounding, as well as wound hormone treatment (JA, ABA, MeJA) and elicitors of defense responses (salicylic acid, arachidonic acid), had no effect on either *ScHT-A* or *S<sub>14</sub>-RNase* mRNA levels (data not shown, except for wounding in Figure 2d). Expression of these genes thus seemed to be exclusively controlled by developmental cues during pistil maturation, except for a differential response toward the type of pollination. *ScHT-A* and *S-RNases* responded differentially to a compatible or an incompatible pollination. In Figure 2(d), flowers were pollinated with either compatible or incompatible pollen and tissues were harvested 48 h later. For the wounding treatment, the upper part of the style including the stigma was slightly crushed with tweezers and tissues were also harvested 48 h later. Following a compatible pollination, or wounding, both *ScHT-A* and *S<sub>14</sub>-RNase* mRNA levels declined similar to



**Figure 2.** RNA expression analysis of *ScHT* transcript levels in styles. (a) Developmental expression pattern of *ScHT-A* and *S<sub>14</sub>-RNase* mRNA levels in unpollinated pistil tissues. *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels were determined by RNA-gel blot analysis of unpollinated pistil tissues, 3 days before anthesis (–3) to 2 days (+2) after anthesis. Ten micrograms of total style RNA from each developmental stage was probed with the *ScHT-A*, cDNA insert, stripped and re-probed with the *S<sub>14</sub>-RNase* cDNA insert. (b) Developmental expression pattern of *ScHT-B* mRNA levels in unpollinated pistil tissues. Same conditions as in (a), except that an identical RNA-gel blot was probed with the *ScHT-B*, specific oligonucleotide. (c) Differential expression of *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels in upper and lower part of the style. *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels were determined by RNA-gel blot analysis in upper and lower halves of styles collected 2 (–2) and 1 day (–1) before anthesis. Conditions same as in (a). (d) Effect of compatible and incompatible pollination on *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels. *ScHT-A* and *S<sub>14</sub>-RNase* transcript levels were determined by RNA-gel blot analysis in unpollinated styles at anthesis day (0), in styles collected 48 h after a fully compatible (*S<sub>11</sub>S<sub>12</sub> × S<sub>13</sub>S<sub>14</sub>*) pollination

the developmentally regulated decrease observed in unpollinated flowers (compare Figure 2a,d). An incompatible pollination had the opposite effect. The *ScHT-A* and *S<sub>14</sub>-RNase* mRNA levels stayed as high as found on anthesis day, indicating that the developmentally programmed decrease in *S-RNase* and *ScHT* mRNA levels could be reversed, at least transiently, following an incompatible pollination.

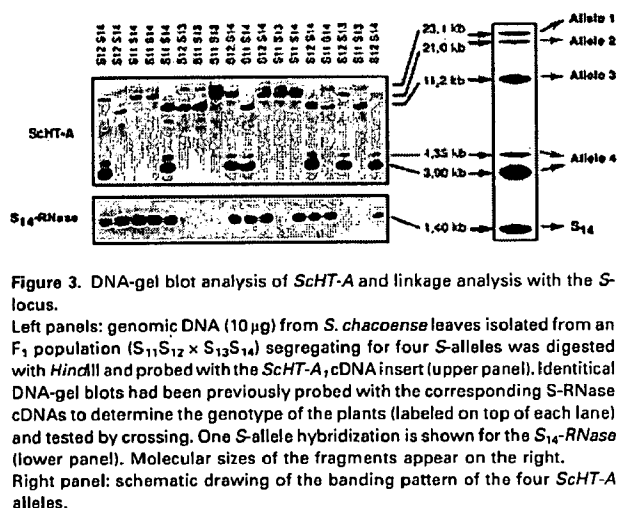
#### Polymorphism of the HT modifiers and linkage to the *S*-locus

Using the *ScHT-A*, cDNA insert as a probe, an F<sub>1</sub> population from a parental cross (*S<sub>11</sub>S<sub>12</sub> × S<sub>13</sub>S<sub>14</sub>*) was tested for polymorphism and linkage to the *S*-locus. A fraction of the F<sub>1</sub> progeny tested is shown in Figure 3. The *S-RNase* genotype of the progeny had been determined previously (Rivard *et al.*, 1994) and was confirmed by PCR analyses with allele-specific primers (data not shown). The *ScHT-A* gene is highly polymorphic as four different RFLPs could be detected in these plants. Although four different *S*-alleles also segregated in this population, the *ScHT-A* alleles were completely unlinked to the *S*-locus, as any combination of *ScHT-A* alleles could be found with all four *S-RNase*s in this population. The same population was re-probed with the *ScHT-B* cDNA. Two new RFLPs specific to the B form were observed (data not shown). Although cross-hybridization does occur between the *ScHT-A* and *ScHT-B* cDNA probes (data not shown), no single RFLP could be linked with the *S-RNase* gene.

#### Two-hybrid analysis of *ScHT* and *S-RNase* protein interaction

A few putative roles have been proposed for the *N. alata* HT protein (McClure *et al.*, 1999). Recently it was shown that *S-RNases*, in both compatible or incompatible interactions, are taken up by pollen tubes, but the entry mechanism is still unknown (Luu *et al.*, 2000). One possibility is that other stylar factors involved in SI, such as the HT protein, could accompany or interact directly with the *S-RNases* as they are being transported into the growing pollen tubes. Since HT proteins from either *S. chacoense* or *N. alata* are fairly acidic proteins with pI around 4, and since *S-RNases* are basic proteins (*S<sub>14</sub>-RNase* mature protein, predicted pI is 9.12), *ScHT* proteins could interact directly with *S-RNases*, albeit not in a sequence-specific manner, as determined by the linkage analysis (Figure 3). Another possibility would be that the HT proteins could interact with the pollen tubes and

**Figure 2.** continued (48/C), in styles collected 48 h after a fully incompatible (*S<sub>13</sub>S<sub>14</sub> × S<sub>13</sub>S<sub>14</sub>*) pollination (48/I) and in styles collected 48 h after wounding (48/W). Conditions same as in (a). To ascertain equal loading conditions, all RNA-gel blots were stripped and re-probed with an 18S ribosomal cDNA probe from *S. chacoense*.

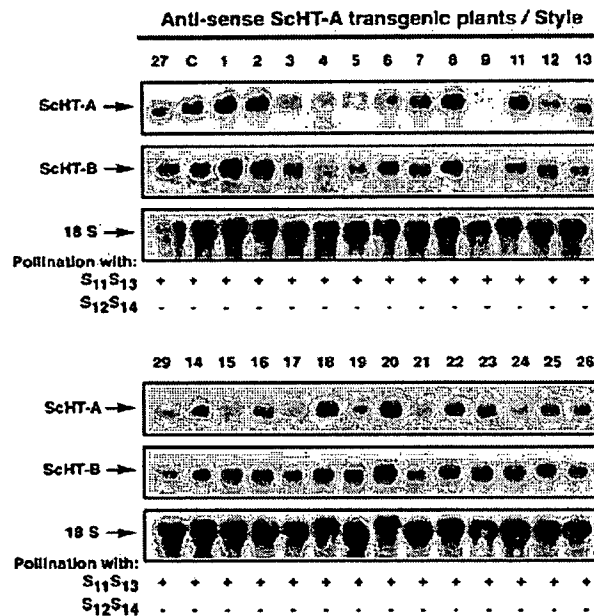


**Figure 3.** DNA-gel blot analysis of *ScHT-A* and linkage analysis with the *S*-locus. Left panels: genomic DNA (10 µg) from *S. chacoense* leaves isolated from an  $F_1$  population ( $S_{11}S_{12} \times S_{13}S_{14}$ ) segregating for four *S*-alleles was digested with *Hind*III and probed with the *ScHT-A* cDNA insert (upper panel). Identical DNA-gel blots had been previously probed with the corresponding *S*-RNase cDNAs to determine the genotype of the plants (labeled on top of each lane) and tested by crossing. One *S*-allele hybridization is shown for the  $S_{14}$ -RNase (lower panel). Molecular sizes of the fragments appear on the right. Right panel: schematic drawing of the banding pattern of the four *ScHT-A* alleles.

facilitate *S*-RNase uptake. To test if the HT protein can interact directly with the *S*-RNase, the *ScHT-A*, and *ScHT-B*, cDNAs were PCR amplified with or without the putative signal peptide, and inserted in frame downstream of the yeast GAL4 DNA-binding domain in the pBDGAL4 vector. Since the linkage analysis (Figure 3) showed that all combinations of *ScHTs* and *S*-RNases could be found in the segregating population, this strongly suggested that no allele-specific interactions would be expected. Thus, a single *S*-RNase gene was used to test putative protein–protein interactions between the *ScHT* and *S*-RNase protein products. A modified  $S_{11}$ -RNase that was previously produced by site-directed mutagenesis was fused to the yeast GAL4 activating domain in the pADGAL4 vector. Because of their intrinsic ribonuclease activity, the  $S_{11}$ -RNase used in the two-hybrid analysis was mutagenized to remove one histidine residue involved in the active site of the enzyme (C3 domain) and replaced with a leucine, thus abolishing the ribonuclease activity that could have prevented proper growth in yeast cells. No direct interaction could be detected between the *ScHT-A* protein, with or without the predicted signal peptide, or the *ScHT-B* protein without the predicted signal peptide and the modified  $S_{11}$ -RNase, as no yeast growth could be observed on histidine-depleted media (data not shown).

#### Molecular characterization of the antisense *Solanum HT-A* plants

To determine if the function of the *ScHT* genes is conserved in solanaceous species other than *N. alata*, antisense *HT-A* plants were produced. The *ScHT-A* cDNA was inserted in the antisense orientation downstream of the CaMV 35S promoter with doubled enhancer in the pBIN19 vector and flanked by the nopaline synthase terminator. *S. chacoense*



**Figure 4.** RNA expression analysis of *ScHT* transcript levels in styles of *ScHT-A* antisense transgenic plants and genetic cross results. *ScHT-A* and *ScHT-B* transcript levels were determined by RNA-gel blot analysis of unpollinated styles at anthesis day from 27 antisense *ScHT-A* transgenic plants. Control plant (C) is the untransformed host (genotype  $S_{12}S_{14}$ ). All crosses were done on at least 10 flowers with pollen from a fully compatible plant (genotype  $S_{11}S_{13}$ ) or a fully incompatible plant (genotype  $S_{12}S_{14}$ ). Ten micrograms of total style RNA from each plant was probed with the *ScHT-A* cDNA insert and the *ScHT-B* specific oligonucleotide. Equal loading conditions were verified with an 18S ribosomal cDNA probe from *S. chacoense*.

plants of  $S_{12}S_{14}$  genotype were transformed with *Agrobacterium tumefaciens* LBA4404 strain containing the *ScHT-A*, antisense construct. Twenty-seven primary transformants were selected. Because *ScHT-A* and *ScHT-B* share 73% nucleotide sequence identity, and since stretches of perfect identity are found between these two sequences, some antisense lines for *ScHT-A* might also be suppressed in *ScHT-B* mRNA accumulation. Figure 4 shows RNA-gel blots of these 27 transgenic plants probed with either the *ScHT-A*, complete cDNA or the *ScHT-B*, specific oligonucleotide, and the results of the genetic crosses with either compatible or incompatible pollen are shown below. In some antisense lines where *ScHT-A* mRNA accumulation had been suppressed, *ScHT-B* levels were also affected, albeit to a lesser extent (AS plant #4 and 9). Although numerous plants showed strong reduction in *ScHT-A* mRNA levels (plants #3–5, 9, 29), none set seeds upon self-pollination.

#### Molecular characterization of the RNAi *Solanum HT-B* plants

Because antisense *S. chacoense HT-A* plants did not become SC, even with an almost 15-fold reduction in *ScHT-A*

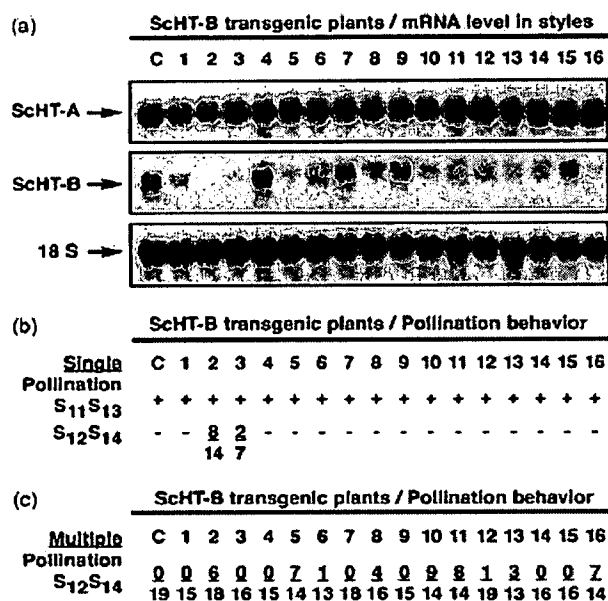


Figure 5. RNA expression analysis of *ScHT* transcript levels in styles of *ScHT-B* RNAi transgenic plants and genetic cross results.

(a) *ScHT-A* and *ScHT-B* transcript levels were determined by RNA-gel blot analysis of unpollinated styles at anthesis day from 16 RNAi *ScHT-B* transgenic plants. Control plant (C) is the untransformed host (genotype S<sub>12</sub>S<sub>14</sub>). Ten micrograms of total style RNA from each plant was probed with the *ScHT-A* cDNA insert and the *ScHT-B* specific oligonucleotide. Equal loading conditions were verified with an 18S ribosomal cDNA probe from *S. chacoense*.

(b) Genetic cross results with pollen from SI or SC plants. All crosses were done on at least 10 flowers per plant on anthesis day, with pollen from a fully compatible plant (genotype S<sub>11</sub>S<sub>13</sub>) or a fully incompatible plant (genotype S<sub>12</sub>S<sub>14</sub>). The plus (+) sign indicates a fully compatible pollination: crosses were successful and seed set occurred in more than 90% of the pollinated flowers. The minus (-) sign indicates a fully incompatible pollination: no crosses were successful and no seed set occurred. For intermediate phenotypes, the number of successful pollination leading to seed set per flower pollinated is indicated.

(c) Genetic cross results with pollen from a SI plant after repeated pollination (multiple pollination). Same as in (b) except that the flowers were repeatedly pollinated with pollen from a fully SI parent (genotype S<sub>12</sub>S<sub>14</sub>). Pollination was done on anthesis day, and then after 24, 48 and 72 h.

transcripts (*ScHT-A* AS plant #9, as determined by densitometric scans), and since correlative evidences showing weak or complete loss of expression of *HT-B* homologues, but not of *HT-A* homologues in some SC *Lycopersicon* species, have been recently obtained (Kondo et al., 2002a,b), we also decided to target the *ScHT-B* gene through an RNA interference (RNAi) strategy. The *ScHT-B* cDNA was inserted first in the sense orientation downstream of the CaMV 35S promoter, followed by a 327-bp spacer, and by the *ScHT-B* cDNA again, but in the antisense orientation. This RNAi construct was then inserted in the *A. tumefaciens* LBA4404 strain and used to transform *S. chacoense* plants of the S<sub>12</sub>S<sub>14</sub> genotype. Sixteen primary transformants were initially selected. All *ScHT-B* RNAi lines were cross-pollinated with pollen from fully compatible

(S<sub>11</sub>S<sub>13</sub>) or fully incompatible (S<sub>12</sub>S<sub>14</sub>) genotypes. Two plants (#2 and #3) sired seeds upon self-pollination (pollen from genotype S<sub>12</sub>S<sub>14</sub>), and could be scored as partially or semi-compatible (Figure 5b). *ScHT-A* and *ScHT-B* mRNA levels were then determined in mature flowers at anthesis. Figure 5a shows an RNA-gel blot of all the transgenic plants probed with either the *ScHT-A* complete cDNA or the *ScHT-B*-specific oligonucleotide, and the results of the genetic crosses with either compatible or incompatible pollen (Figure 5b). Unlike the *ScHT-A* antisense experiment (Figure 4), the RNA interference strategy specifically targeted the *ScHT-B* transcript as no significant variation in the *ScHT-A* mRNA levels could be observed (Figure 5a). Only the transgenic plants with the most reduced *ScHT-B* mRNA level became partially SC (plants #2 and 3), suggesting that a threshold level of *ScHT-B* is necessary to maintain the SI phenotype, and that only the HT-B isoform is involved in GSI.

#### *The ScHT-B gene affects flower longevity and stylar abscission following an incompatible pollination*

One intriguing observation, following an incompatible pollination, was that flowers of *ScHT-B* RNAi plants that had lower levels of *ScHT-B* transcripts, stayed much longer on the plant than control or transgenic plants not affected in *ScHT-B* mRNA levels (plants #4, 7, 9 and 15). Under normal conditions, abscission of unpollinated flowers in *S. chacoense* occurs approximately 5 days after anthesis (Figure 6, unpollinated G4). After a compatible pollination, ovary swelling is clearly detectable 3 days after pollination and stylar abscission occurs approximately 4 days after pollination (Figure 6, V22 × G4). Following an incompatible pollination, abscission is delayed by an average of 24 h when compared to unpollinated flowers (Figure 6, G4 × G4). In *ScHT-B*-suppressed lines, flower abscission was further delayed and only occurred after an 8–9-day period following initial pollination (data not shown). This extended flower longevity phenotype caused by a lower than normal *ScHT-B* mRNA level prompted us to re-examine pollination behaviour with SI pollen under a multiple pollination scheme. In this experiment, *ScHT-B* transgenic plants and untransformed control plants were pollinated on anthesis day, and then on the following 3 days with similar pollen load. Fruit formation was then monitored from day 6 to 12 after pollination. As a control, the transformation host genotype was also repeatedly pollinated. Even after multiple pollination, the untransformed plant (control) and the transgenic plants not affected in *ScHT-B* levels (plants #4, 7, 9 and 15) never sired seeds, indicating that multiple pollination alone, even over a 72-h period, was not sufficient to bypass the SI recognition and rejection system (Figure 5c). For the remaining 12 transgenic plants with altered level of *ScHT-B* mRNA, a total of nine plants were scored as

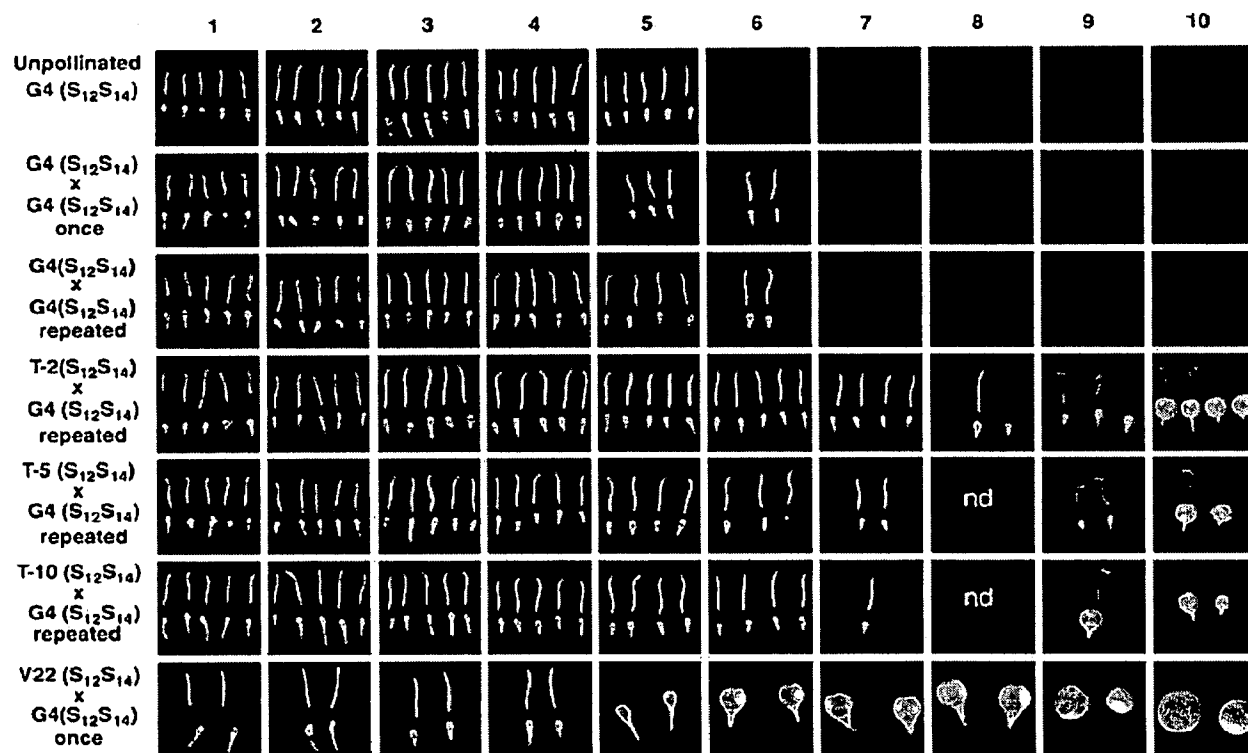


Figure 6. Pistil morphology of *ScHT-B* transgenic and control plants after SI and SC pollination. Floral and stylar abscission was monitored, from one day after anthesis or from one day after pollination for control plants, and for three transgenic plants (T-2, T-5 and T-10) until day 10. In all cases, pollination was performed with pollen from the G4 line (genotype  $S_{12}S_{14}$ ). Five flowers per plant and per day (except for the  $V22 \times G4$  cross) were hand pollinated on consecutive days and, for the remaining flowers, dissected at the end of the time-course. Only the remaining floral parts at the end of the time-course are displayed. ND, not determined. Once, single pollination. Repeated, consecutive pollination on day 0–2 and if possible on day 3, depending on the corolla closure.

semi-compatible (plants #2, 5, 6, 8, 10–13 and 16) upon repeated pollination with fully incompatible pollen (genotype  $S_{12}S_{14}$ ). Floral and stylar abscission were also monitored, from one day after anthesis or from one day after pollination for control plants and three transgenic plants (T-2, T-5 and T-10) that showed a SC behaviour following self-pollination. Five flowers per plant and per day were hand pollinated on consecutive days in order to collect all samples on the same day (except for the unpollinated control plant and the fully compatible cross  $V22 \times G4$ ). As such, with the remaining flowers at the end of the 10-day period, the whole time-course was displayed. Pistil morphology for these plants is shown in Figure 6. Transgenic plants, T-2, T-5 and T-10, clearly showed an increased stylar longevity, with turgid styles that appeared receptive to pollination until day 7 or 8, after pollination. Furthermore, stylar abscission from the developing fruit was also delayed, with some styles still attached even after withering (Figure 6, plants T-2 and T-5 on day 9 and 10; plant T-10 on day 9). When compared to a fully compatible cross ( $V22 \times G4$ ), fruit formation was also delayed in self-pollinated T-2, T-5 and T-10 transgenic plants. These results

confirm the involvement of the *ScHT-B* gene in SI and suggest that it might act through an increased flower receptivity period.

## Discussion

Mechanisms underlying the breakdown of GSI have been recently reviewed and grouped in three broad categories (Stone, 2002). First, loss of SI occurs following the duplication of the *S*-locus and the presence of heterozygous pollen (heteroallelic for the *S*-locus) (Golz *et al.*, 2000). Secondly, mutations affecting either the expression of the *S*-RNase or its activity also lead to a SC phenotype (Royo *et al.*, 1994). Thirdly, mutations not affecting the enzymatic activity of the *S*-RNase have also been described at the genetic level and include many so-called modifier loci. Numerous experiments have demonstrated that although the *S*-RNase is responsible for pollen recognition and rejection in the style (Lee *et al.*, 1994; Matton *et al.*, 1997; Matton *et al.*, 1999; Murfett *et al.*, 1994), other stylar factors are also necessary for the proper expression of the SI phenotype (Ai *et al.*, 1991; Bernatzky *et al.*, 1995; Kondo *et al.*, 2002b; Murfett

*et al.*, 1996). Such factors, often considered as modifier loci, are present in the genetic background of SI plants, unlinked to the *S*-locus, and have often been lost in SC relatives of SI species. Complementation phenomena of the genetic background have been described in *L. hirsutum* where the  $F_1$  population from two independent SC accessions were all SC, while SI offsprings could be recovered in the  $F_2$  generation from these  $F_1$  plants, strengthening the multigenic nature of the gametophytic SI (Ricks and Chetelat, 1991). One such candidate for a modifier gene is the *N. alata* HT gene (McClure *et al.*, 1999). The *NaHT* gene was cloned based on a differential screen between stylar expressed mRNAs from SC *N. plumbaginifolia* and an SC accession of *N. alata* that is defective in S-RNase expression but that is competent to express SI (Murfett *et al.*, 1996). Anti-sense *NaHT* plants with reduced level of the HT protein but with normal levels of S-RNases were either fully or partially SC (McClure *et al.*, 1999). This strongly suggests that the *NaHT* gene is a good candidate for such a modifier factor necessary for the SI reaction to occur. In the present study, we have characterized *NaHT* homologues from four different *Solanum* species, and have focussed our attention on the putative function of the *S. chacoense* HT homologues (*ScHT-A* and *ScHT-B*) in GSI.

Phylogenetic analyses of the isolated *NaHT* homologues clearly demonstrated that two different HT isoforms exist and that isoform B is probably the most closely related to the *NaHT* gene. All the HT proteins shared some common features. Firstly, a highly conserved N-terminal region that is strongly predicted to be a signal peptide. The sequence conservation was high enough to originally derive PCR primer pairs from only the *NaHT* and *ScHT-A* sequences, and amplify both HT isoforms from numerous *Solanum* (this study) and *Lycopersicon* species (Kondo *et al.*, 2002a,b). Secondly, all HT homologues possess a C-terminal region composed of consecutive stretches of asparagine and aspartic acid residues, flanked by conserved cysteines probably involved in disulfide bridges. Although sequence identity is quite variable, ranging from 36 to 92% in the B-isoform group (77–92% when only *Solanum* sequences are considered), the overall structure conservation combined with identical expression pattern would suggest that the *ScHT-B* isoform and the *NaHT* protein are probably true orthologues. Both *ScHT-A*, *ScHT-B* and *NaHT* are almost exclusively stylar-expressed as for the S-RNases, and all are developmentally regulated during pistil maturation (this study and McClure *et al.*, 1999). Interestingly, we found higher expression levels of both *ScHT* and *S-RNase* genes in the upper style region (Figure 2c), consistent with the pattern of pollen tube arrests that occurs in the top half of the style in *S. chacoense* (Matton *et al.*, 1999). The developmental regulation of S-RNase transcripts accumulation enables the production of selfed progeny in some GSI species when using very young flower buds (bud-

pollination), but is very difficult to achieve in *S. chacoense*. One reason could be the elevated level of both S-RNase and HT transcripts, even 2 days before anthesis, and detectable 3 days before anthesis, combined with a preferential upper style accumulation. One intriguing observation was the differential expression pattern of both *S-RNase* and *HT* transcripts following an incompatible pollination compared to an unpollinated or a compatibly pollinated flower (Figure 2d). As the flower ages, *S-RNase* and *HT* transcript levels decreases markedly, but low expression levels coincide with reduced fertilization receptivity, and eventually, flower abscission. Surprisingly, *S-RNase* and *ScHT-A* transcript levels do not decrease following an incompatible pollination (for at least 2 days after pollination, Figure 2d), and this cannot be the result of only stigmatic and transmitting tissue deterioration and death, since this is also induced by a compatible pollination. Furthermore, mechanical wounding or wound hormone treatments had no effect on *S-RNase* and *ScHT-A* transcript levels. This strongly suggests that the presence of dead pollen tubes or molecules liberated from the arrested pollen tubes, either increase the transcription of these genes, or reduce their mRNA turnovers, ensuring that the S-RNases and HT proteins are still present in sufficient amount to reject newly incoming pollen from incompatible genotypes. The maintenance of high steady-state levels of *S-RNases* and *ScHT* mRNAs following an initial incompatible pollination, would also lead to a prolonged reproductive barrier, an important issue since flower senescence is retarded following an incompatible pollination (Figure 6, G4 × G4).

In order to determine the role of the *ScHT* genes in SI, functional analysis of *ScHT-A* and *ScHT-B* protein–protein interactions with an S-RNase were tested in the yeast two-hybrid system, and transgenic plants with strongly suppressed levels of both isoforms were generated. Although *ScHT-A* and *ScHT-B* deduced mature proteins have acidic pIs and the S-RNase is basic (predicted pI = 9.25 for *S<sub>11</sub>*-RNase mature protein), no direct interactions based either on specific or electrostatic attractions could be detected in the two-hybrid system, as no yeast growth could be observed, with or without the predicted signal peptide. Such direct interaction had also not been detected with the purified HT protein from *N. alata*, although in that case, the *NaHT* protein appeared to be unstable in stylar extracts (McClure *et al.*, 1999). Both results suggest that HT proteins and S-RNases do not interact directly.

Recently, correlative evidences for the involvement of the *NaHT* homologues in *Lycopersicon* species have been obtained (Kondo *et al.*, 2002a,b). In the three *Lycopersicon* SI species tested, all expressed functional S-RNases as well as *HT-A* and *HT-B* mRNAs. In the seven *Lycopersicon* SC species tested, no or low stylar ribonuclease activity was observed. This alone would most probably be sufficient to explain their SC phenotype, since a threshold level of

S-RNase expression is necessary to confer an SI phenotype (Lee *et al.*, 1994; Matton *et al.*, 1997). Intriguingly, in the seven *Lycopersicon* SC species tested, transcription of the HT-B isoform was either weakly or not detected at all, and the HT-B isoform produced had internal stop codons, while the HT-A isoform was strongly expressed at the mRNA level, although some SC species also produced defective (frame-shifted) HT-A transcripts. Apart from the *N. alata* transgenic antisense lines, no other functional analysis had been made prior to the one presented here. In *N. alata*, plants with reduced levels of the NaHT protein were either fully or partially SC, suggesting that the amount of NaHT protein is important (McClure *et al.*, 1999). In the present study, antisense *ScHT-A* and RNAi *ScHT-B* plants were generated. Figure 4 showed that even with a 15-fold decrease in *ScHT-A* mRNA levels, *ScHT-A* AS plant #9 remained SI. *ScHT-B* mRNA levels in that transgenic line were also affected, although to a lesser extent. This could suggest that a threshold level of *ScHT-B* is sufficient to maintain an SI phenotype. In a second series of experiments, *ScHT-B* suppression was achieved through an RNAi strategy. Unlike the antisense plants, the *ScHT-B* RNAi plants were only affected in *ScHT-B* mRNA expression (Figure 5). Plants with severely reduced *ScHT-B* transcripts became SC and sired seeds upon pollination with pollen from an incompatible genotype. RNAi plant #2 had the most severely reduced *ScHT-B* mRNA levels and consistently set seeds upon self-pollination. RNAi plant #3 had a less stable phenotype, and only sired seeds occasionally. Although at first only two plants became partially SC upon selfing, all the transgenic plants with reduced *ScHT-B* mRNA levels also showed an extended, albeit slightly variable, floral longevity upon pollination with incompatible pollen. This observation led to the hypothesis that the *ScHT-B* gene could be involved in modulating the receptivity period of the flower, perhaps through a control over the abscission of the floral organs. This increase in floral longevity, and in particular in stylar turgescence and receptivity might partially explain the SC phenotype since it could increase the chances of pollen tubes to reach the ovary. To test this, repeated pollination were performed on 3–4 consecutive days, on the 16 RNAi *ScHT-B* transgenic plants and control plants. None of the *ScHT-B* transgenic plants unaffected in *ScHT-B* mRNA expression, or the untransformed control plant, had an extended floral longevity and none were fertilized upon selfing. Of the remaining 12 transgenic plants expressing reduced levels of *ScHT-B* mRNA, nine were able to sire seeds. Fruits formed on these plants were smaller than the ones obtained from a compatible cross, and after 10 days, were comparable in size with fruits produced from a compatible pollination after 6 days (Figure 6, compare the fruits from plants T-2, T-5 and T-10 with the ones from the V22 × G4 cross). These results are entirely consistent with our hypothesis that reduced level of *ScHT-B* mRNA affects

the receptivity period of the flower and that the SC phenotype observed in *ScHT-B* transgenic plants does not result only from the developmentally regulated decrease in both *S-RNase* and *ScHT-B* mRNA levels (Figure 2a,b), since repeated incompatible pollination could not induce fertilization in control or unaffected *ScHT-B* transgenic plants. Furthermore, in *N. alata* HT antisense plants, pollen tube growth in the style is observed even when the S-RNase level is high, although in that case, fertilization and production of fruits could not be observed because the recipient plant used was a sterile hybrid between *N. alata* and *N. plumbaginifolia* and only pollen tube growth in the style was used to score the SI or SC phenotype.

Our results clearly indicate that there is an increase in flower longevity and pollination receptivity, associated with a decrease in *ScHT-B* transcripts. One possibility would be that the HT-B isoform is involved in a pathway regulating floral abscission. Pollination is known to affect the physiological state of the flower. Pollinated flowers (compatible) senesce rapidly compared to unpollinated flowers or those pollinated by incompatible pollen grains in the case of an SI plant (Gilissen, 1977; Singh *et al.*, 1992). Early studies in *Petunia* ovaries showed an increase in polyribosomes activity, 6–12 h after pollination, well before the arrival of the pollen tubes in the ovary (approximately 50 h) (Deurenberg, 1976). Pollination-induced wilting of the corolla can be prevented if the style is removed early after pollination (Gilissen, 1984). These and other results (Stead, 1992) have led to the hypothesis that a pollination-induced signal is transmitted through the pistil and precedes the growing pollen tube. Ethylene has been shown to have a strong effect on flower abscission in solanaceous species (van Doorn, 2002a,b). Furthermore, pollination itself induces ethylene synthesis (Hall and Forsyth, 1967), and it has been shown that in *P. hybrida*, pollination induces two distinct phases of ethylene production in the flower (Singh *et al.*, 1992). The first phase is common to both self- and cross-pollinated flowers and is dependent on pollen-borne ACC (ethylene precursor). The second phase results from *de novo* synthesis of ethylene from the flower and occurs 18 h after a compatible pollination. Following an incompatible pollination, the production of ethylene is delayed to 3 days after pollination (Singh *et al.*, 1992). Since RNAi *ScHT-B* plants showed delayed floral abscission, we tested these plants for alteration in the expression of ethylene-related genes. No differences could be observed in the expression pattern of two genes involved in ethylene biosynthesis (ACC synthase and ACC oxidase), or in ethylene perception and signal transduction (ethylene receptor ETR1 and EIL-3) in *ScHT-B* transgenic plants (data not shown). Since the ACC synthase and the ACC oxidase genes are part of multigene families (at least eight members for the ACC synthase and four members for the ACC oxidase in *S. lycopersicon*) (Llop-Tous *et al.*, 2000), specific probes will need



to be designed for individual members in order to determine if a given isoform is affected in *ScHT-B* mutant background.

From our results, we propose that the *ScHT-B* isoform is involved in at least two phenomena. Firstly, elevated levels of both *S-RNases* and *ScHT-B* would be necessary for the SI reaction to occur, as determined from McClure's work (McClure *et al.*, 1999) and from the phenotype of the *ScHT-B* RNAi plant T-2. When the *ScHT-B* mRNA levels are below a threshold level, pollen tubes would be able to reach the ovary and effect fertilization. The developmentally regulated decrease in both *S-RNase* and *ScHT* mRNA levels (Figure 2a,b) would normally lead to an SC phenotype in aged flowers, but is counterbalanced by floral abscission. The increase in both *S-RNase* and *ScHT* mRNA levels following an incompatible pollination (Figure 2d) would also ensure the maintenance of a strong reproductive barrier over a longer period of time. This could be of importance since flowers pollinated with incompatible pollen last longer by an average of 1 day on the plant than unpollinated flowers (Figure 6, G4 × G4), and the receptivity period for a successful pollination is normally limited to the first 2–3 days after anthesis. Secondly, the *ScHT-B* RNAi transgenic plants display a novel phenotype that includes a longer floral longevity with delayed stylar abscission and, perhaps, more relevant for the SC phenotype of those plants, the persistence of turgid styles, even 9 days after anthesis (Figure 6). This phenotype, observed in plants with reduced levels of *ScHT-B* mRNAs, would enable pollen tube growth in older styles with reduced *S-RNase* level, pass their normal flower lifespan and pollination receptivity period. As suggested by McClure (McClure *et al.*, 1999), the HT protein could interact directly with pollen tubes and facilitate *S-RNase* uptake. Our two-hybrid results would support the fact that *ScHT-B* does not interact directly with the *S-RNase*. Furthermore, if the HT-B protein is involved in *S-RNase* uptake, a reduced level of HT-B protein would increase the number of pollen tubes not affected by the presence of the *S-RNase*, enabling fertilization to take place. This is entirely consistent with our results, since repeated pollination in *ScHT-B* RNAi plants increase the percentage of fertilized ovules. Although its mode of action still remains unclear, our data demonstrate a specific role for the HT-B isoform in SI and points towards a role in the control of flower senescence and abscission.

## Experimental procedures

### Plant material and transformation

The diploid ( $2n=2x=24$ ) *S. chacoense* Bitt. SI genotypes used included line 314 ( $S_{11}S_{12}$ ), 582 ( $S_{13}S_{14}$ ), G4 ( $S_{12}S_{14}$ ) and V22 ( $S_{11}S_{13}$ ). Plants were grown in greenhouse with 14–16 h of light per day. Transformation was done as described previously and the

transformation host plant was line G4 (Matton *et al.*, 1997). The *ScHT-A* cDNA was cloned in antisense orientation downstream of a CaMV 35S promoter with doubled enhancers (Skuzeski *et al.*, 1990) and flanked by the nos terminator in the pBIN19 vector (Bevan, 1984). For RNA interference experiments, a new vector was constructed. This new vector, called pDARTH (O'Brien and Matton, unpublished), includes a CaMV 35S promoter with doubled enhancers (Skuzeski *et al.*, 1990), an extended multiple cloning site and a 327-bp intron from a histone deacetylase (*HD2*) gene from *S. chacoense* (our unpublished results). The *ScHT-B* cDNA sequence (324 bp) was cloned in sense and antisense orientation separated by the *HD2* intron.

### Isolation of the *ScHT* cDNAs and PCR amplification of other solanaceous HT genes

The *ScHT-A*<sub>1</sub>, *ScHT-A*<sub>2</sub> and *S*<sub>14</sub>-*RNase* cDNAs were initially isolated from a pollinated pistil cDNA library using virtual subtraction (Li and Thomas, 1998). In this procedure, genes corresponding to low-expressed mRNA species are preferentially isolated. Because the initial screen was for rare mRNA species expressed in ovary tissues, and since the library also contained cDNAs expressed in styles, genes that were highly expressed in styles but only weakly expressed in ovaries were also recovered. A second screening round with a probe derived from stylar mRNAs, uncovered all of the stylar expressed genes, including the *ScHT-A*<sub>1</sub>, *ScHT-A*<sub>2</sub> and *S*<sub>14</sub>-*RNase* cDNAs. For the isolation of the *ScHT-B*<sub>1</sub> cDNA and of related sequences in other solanaceous species, three degenerate primers were designed based on the most conserved amino acid sequence of *ScHT-A*<sub>1</sub> from *S. chacoense* and HT from *N. alata* (McClure *et al.*, 1999). The sequence of the upstream primers (HT-NS1: 5'-TTT CTT TGG TTC TT(A/T) TGA T(A/T)A TAT CAT CA-3'; HT-NS2: 5'-ATA TCA TCA GA(A/G) GTT ATT GC(A/T) AGG GA(A/T) ATG-3') are derived from the predicted signal peptide sequence, and the sequences of the downstream primers (HT-C1: 5'-TCC TTT ATT CAA CCA AT(C/T) TCA TAT TA-3'; HT-C2B: 5'-CAA AAA TAT TAC ATA ATA TTT TGT AGT CG-3') are derived from the C-terminus of the HT protein. The *S. chacoense* HT-B1 isoform was obtained by PCR amplification of cDNAs from a pollinated pistil library while HT isoforms from *S. pinnatisectum*, *S. bulbocastanum* and *S. tuberosum* were obtained by PCR amplification of genomic DNA.

### Isolation and gel blot analysis of RNA and DNA

Total RNA was isolated as described previously (Jones *et al.*, 1985). RNA concentration was determined by measuring its absorbance at 260 nm and verified by agarose gel electrophoresis following ethidium bromide staining. To confirm equal loading of total RNA on RNA-gel blots, a 1-kb fragment of the *S. chacoense* 18S rRNA was PCR amplified and used as a probe (Lantin *et al.*, 1999a). Genomic DNA isolation was performed via a modified CTAB extraction method (Reiter *et al.*, 1992) or with the Plant DNeasy kit from Qiagen. DNA-gel blot analysis, including restriction, electrophoresis and capillary transfer to a positively charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Baie D'Urfé, Québec) were performed as described previously (Sambrook *et al.*, 1989). Hybridization of the membrane was performed under high stringency conditions at 65°C as described previously (Church and Gilbert, 1984) for 16–24 h, and following hybridization, the membrane was washed at room temperature twice with 2X SSC/0.1% SDS for 30 min, twice with 1X SSC/0.1% SDS at 50°C for 30 min and twice with 0.1X SSC/0.1% SDS at 55°C for 10 min (1X SSC is 0.15 M

NaCl, 0.015 M sodium citrate, pH 7.0). RNA-gel blot analyses were performed as described in Sambrook *et al.* (1989), following the formaldehyde denaturing protocol. RNAs were capillary transferred to Hybond N+ nylon membranes and cross-linked ( $120 \text{ mJ cm}^{-2}$ ) with a Hoefer UVC 500 UV Crosslinker. Hybridization of the membranes was performed under high stringency conditions at  $45^\circ\text{C}$  in 50% deionized formamide, 5X Denhardt solution, 0.5% SDS,  $200 \mu\text{g ml}^{-1}$  denatured salmon sperm DNA and 6X SSC for 16–24 h. Following hybridization, the membranes were washed at room temperature twice with 2X SSC/0.1% SDS for 30 min, twice with 1X SSC/0.1% SDS at  $50^\circ\text{C}$  for 30 min and twice with 0.1X SSC/0.1% SDS at  $55^\circ\text{C}$  for 10 min. Probes for DNA-gel blot analysis were synthesized from random-labeled isolated DNA inserts (Roche Diagnostic, Laval, Québec) with  $\alpha\text{-}^{32}\text{P}$  dCTP (ICN Biochemicals, Irvine, CA). For RNA-gel blot analyses, cDNA probes were made with  $\alpha\text{-}^{32}\text{P}$  dATP with the Strip-EZ DNA labeling kit (Ambion, Austin, TX) and oligonucleotide probes were labeled with  $\gamma\text{-}^{32}\text{P}$  dATP (Sambrook *et al.*, 1989). The membranes were autoradiographed at  $-85^\circ\text{C}$  with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, Ontario).

#### Site-directed mutagenesis of the *S<sub>11</sub>*-RNase and yeast two-hybrid analysis

A mutated *S<sub>11</sub>*-RNase gene with the conserved His-114 residue (CAT) located in the C3 active site domain was converted to a leucine residue (CTT) by site-directed mutagenesis using the following oligonucleotide (mutated nucleotide is underlined): 5'-CTAAAGCTTGGATCCTGCTGT-3' (Altered sites II *in vitro* mutagenesis system, Promega, WI). The original construct contained both the *S<sub>11</sub>* intron and 3' end of the gene, and was expressed in transgenic *S. chacoense* plants (Matton *et al.*, 1997; Matton *et al.*, 1999) under the control of the style specific chitinase promoter (Harikrishna *et al.*, 1996). The spliced His<sup>+</sup> *S<sub>11</sub>*-RNase cDNA was recovered from reversed transcribed style mRNAs, and the coding region corresponding to the mature protein was PCR amplified (Pwo DNA polymerase, Roche Diagnostics, Laval, Québec) and fused in frame with the DNA-binding domain of the GAL4 protein in the pBDGAL4 yeast vector (TRP1 selection marker) (Stratagene, LaJolla, CA). The *S<sub>11</sub>*-A coding region was PCR amplified with or without the predicted signal peptide and inserted in frame with the GAL4 activation domain in the pADGAL4 vector (LEU2 selection marker). For the *S<sub>11</sub>*-B construct, only the coding region without the predicted signal peptide was inserted in frame with the GAL4 activation domain in the pADGAL4 vector. Integrity of the DNA constructs was verified by sequencing. The constructs were transformed sequentially in the yeast strain PJ69-4A (James *et al.*, 1996) and selected through their ability to grow on Trp<sup>+</sup> and Leu<sup>+</sup> media. Protein-protein interaction assays were performed on media lacking Trp, Leu and His and on media lacking Trp, Leu and Ade.

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GenBank accession numbers: *ScHT-A<sub>1</sub>* (AF442139), *ScHT-A<sub>2</sub>* (AF442140), *ScHT-B<sub>1</sub>* (AF442141), *StHT-A<sub>1</sub>* (AF442142), *SbHT-B<sub>1</sub>* (AF442143), *SbHT-B<sub>2</sub>* (AF442144), *SpHT-B<sub>1</sub>* (AF442145), *SpHT-B<sub>2</sub>* (AF442146), *LpHT-A<sub>1</sub>* (AB066582), *LpHT-B<sub>1</sub>* (AB066583), and *S<sub>14</sub>-RNase* (AF232304).



# Exhibit 20

Patent  
Attorney's Docket No. 021565-060

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
Peter Waterhouse et al.	)	Group Art Unit: 1635
Application No.: 09/287,632	)	Examiner: ZARA JANE
Filed: April 7, 1999	)	Confirmation No.: 6526
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED	)	
PHENOTYPES	)	

### Declaration of Dr. Marc De Block under 37 C.F.R. section 1.132

I, Marc De Block, hereby declare that:

1. I am a citizen and resident of Belgium. I received a Ph. D. degree in 1981 from the University of Ghent, Belgium.
2. Since 1984, I have been employed in Ghent, Belgium by PLANT GENETIC SYSTEMS N.V. or its successor firms AVENTIS CROPSOURCE N.V. and BAYER BIOSOURCE NV. My work has involved the supervision of projects studying plant growth and development as well as supervision of projects studying tissue specific gene-silencing.
3. I am familiar with the field of the plant molecular biology, particularly the fields of cell cycle research, plant development, apoptosis and stress resistance and I have authored and co-authored several scientific publications in these fields (a list of publications is submitted as ANNEX 1). I am also familiar with the field of post-transcriptional gene silencing and have applied such technology to achieve various traits as described in several scientific publications, e.g. those listed in ANNEX 1 as items 24 and 25.
4. I have read US Patent Application No. 09/287,632 (the "Application") including the presently pending claims.
5. I have been informed that Bayer BioScience N.V. has obtained a license to the inventions as described e.g. in the Application.
6. I have further been informed that in the Office Action dated 8 February 2007 issued by the United States Patent and Trademark Office in connection with the Application, the Examiner alleged that "The claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention."

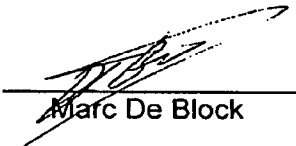
7. It is my understanding that the Examiner believes that the specification, claims and the art do not adequately describe the distinguishing features or attributes shared by the members of the claimed genus of DNA constructs for gene silencing comprising the recited sense and antisense nucleotide sequences and further comprising an intron sequence, whereby any intronic sequence is inserted anywhere in the chimeric DNA, and whereby the DNA construct provides for the function claimed, namely reducing the phenotypic expression of a nucleic acid of interest in any plant cell or in any isolated eukaryotic cell.
8. The Examiner also asserts that the Application provides only two examples of chimeric constructs which contain an intron. Thus, the Examiner believes that the Application does not describe a sufficient number of different possibilities for the chimeric dsRNA constructs claimed to allow a person skilled in the art to conclude that the inventors were in possession of the genus as broadly claimed.
9. I disagree with the Examiner for the following reasons.
10. In the period of 1999-2003, I supervised and participated in a project at Bayer BioScience N.V., together with Dr Marina Byzova aiming at reducing the adsorption or reflection of photosynthetically active radiation by the bright yellow flowers of oilseed rape. We investigated whether hairpin RNA-mediated gene silencing could be implemented to silence B-type MADS-box floral organ identity genes in a tissue-specific manner (i.e. in the second whorl only) to transform the yellow petals into green sepaloid structures in Arabidopsis and oilseed rape. The results of this project are summarized in the peer-reviewed publication listed as item 24 in my publication list and attached to this declaration as ANNEX 2.
11. The design of the hairpin RNA encoding chimeric genes is described in detail on page 380 right column of ANNEX 2, in the section entitled "Plasmid construction". The hairpin RNA encoding chimeric genes contained either a 3' end of the *B. napus* homologue of APETALA3 ("AP3") or of the PISTILLATA ("PI") gene of *A. thaliana*, cloned in inverse orientation so that upon transcription a double stranded RNA molecule or hairpin RNA molecule could be formed. Both AP3 and PI are examples of B-type MADS-box floral organ identity genes.
12. At the time of designing the hairpin RNA encoding chimeric genes we were aware --due to our collaboration with CSIRO, assignee of the Application-- of the results indicated in the Application at least on page 23 and in Example 6 that the inclusion of an intron in the transcribed region enhances the efficiency of the reduction of expression of the gene of interest. Therefore, we decided to include an intron in at least one of the chimeric constructs (indicated in ANNEX 2 as pAP1::hpBPI) that were to be used in the project.
13. Since I did not perceive the identity of the particular intron to be used to be critical to achieve the same effect, another intron which was readily available at our premises was used i.e. intron IV2 from the potato light-inducible tissue-specific gene ST-LS1 as described by Vancanneyt et al., 1990, Mol. Gen. Genet. 220:

245-250 (see ANNEX 2, page 380, 8-9 lines from the bottom of the right-hand column).

14. Interestingly, the hairpin RNA encoding construct comprising the intron (pAP1::hpBPI) is the most efficient in Arabidopsis to achieve the desired double sepeloid phenotype (ANNEX 2, page 381 right hand column lines 26-27) and is the only construct when used in oilseed rape resulting in flowers with an apetalous or partially apetalous phenotype, while no differences in flowering were observed with the intron-less construct (ANNEX 2, paragraph spanning pages 382 and 383). Further modifications of the promoter driving the intron comprising hairpin RNA constructs finally resulted in the oilseed rape with sepeloid petals.
15. I believe the above described clearly illustrates that the invention described in the Application was in my mind not limited to the particular exemplified intron, as I immediately decided to exchange it for another known intron. I believe that a person of ordinary skill in the art reading the Application at the time the Application was filed would have reached a similar understanding.
16. In my view a person of ordinary skill in the art for the purpose of the inventions described in the Application is a person with a doctoral degree in the field of molecular biology of eukaryotes with at least some years of post doctoral research experience.
17. It is also my opinion that a person of ordinary skill in the art would have immediately understood that the applicability of the inventions described in the Application is not limited to the specific Example therein, nor to the particular intron used in that Example. The person of ordinary skill in the art would have understood that the exemplified intron could be exchanged for other well known introns, and that such other introns would function in the same manner, since the process of removal of introns from RNA molecules is a conserved process.
18. In conclusion, it is therefore my opinion that a person of ordinary skill in the art would have reasonably concluded, judged at the filing date, that the Application adequately described the claimed subject matter, to convey that the inventors were in possession of the invention as broadly claimed.

I also declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of this application or any patent issued thereon.

06/08/07  
Date

  
Marc De Block



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ORIGINAL ARTICLE

Marina Byzova · Christoph Verduyn · Dirk De Brouwer  
Marc De Block

## Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner

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**Abstract** Oilseed rape (*Brassica napus* L.) genotypes with no or small petals are thought to have advantages in photosynthetic activity. The flowers of field-grown oilseed rape form a bright-yellow canopy that reflects and absorbs nearly 60% of the photosynthetically active radiation (PAR), causing a severe yield penalty. Reducing the size of the petals and/or removing the reflecting colour will improve the transmission of PAR to the leaves and is expected to increase the crop productivity. In this study the 'hairpin' RNA-mediated (hpRNA) gene silencing technology was implemented in *Arabidopsis thaliana* (L.) Heynh. and *B. napus* to silence B-type MADS-box floral organ identity genes in a second-whorl-specific manner. In *Arabidopsis*, silencing of B-type MADS-box genes was obtained by expressing *B. napus* *APETALA3* (*BAP3*) or *PISTILLATA* (*BPI*) homologous self-complementary hpRNA constructs under control of the *Arabidopsis* A-type MADS-box gene *APETALA1* (*API*) promoter. In *B. napus*, silencing of the *BPI* gene family was achieved by expressing a similar hpRNA construct as used in *Arabidopsis* under the control of a chimeric promoter consisting of a modified petal-specific *Arabidopsis* *AP3* promoter fragment fused to the *API* promoter. In this way, transgenic plants were generated producing male fertile flowers in which the petals were converted into sepals (*Arabidopsis*) or into sepaloid petals (*B. napus*). These novel flower phenotypes were stable and heritable in both species.

**Keywords** Apetalous · *Arabidopsis* · *Brassica* · Double sepaloid · MADS-box · Petal

**Abbreviations** PAR: photosynthetically active radiation · *ST-LS1*: potato light-inducible tissue-specific *ST-LS1* gene · *GUS*:  $\beta$ -glucuronidase

### Introduction

Flowers of oilseed rape (*Brassica napus*) have four well-developed bright-yellow petals. During flowering time, flowers form a very bright-yellow layer that reflects and absorbs solar radiation. As consequence, only 24% of the photosynthetically active radiation (PAR) reaches the leaf canopy (Chapman et al. 1984). This accelerates leaf and bract senescence, reduces dry matter accumulation, and lowers seed set (Daniels et al. 1986).

A few strategies to improve the photosynthetic efficiency of oilseed rape by utilising different apetalous variants (Buzza 1983; Jiang and Becker 2003) or the *stamenoid petal* (stap) variant with flowers bearing staminoid petals (Fray et al. 1997) have been proposed. Physiological analyses have revealed the potential benefit of such a petalless flower phenotype on *B. napus* yield (Rao et al. 1991; Fray et al. 1995).

The currently used apetalous genotypes are controlled either by two recessive genes (Fray et al. 1996) or by an interaction of cytoplasmic genes and two pairs of nuclear genes (Jiang and Becker 2003). This genetic complexity makes it difficult to fully implement the apetalous trait into commercial rapeseed varieties. Additionally, the apetalous character appears to be unstable under field conditions at high temperatures and in long days (Rao et al. 1991). The *B. napus* *stap* variant also possesses poor agronomic attributes, such as deformed leaves and poor vigour (Fray et al. 1997).

A more promising strategy to improve PAR transmission in oilseed rape would be the use of a single dominant gene that converts the bright-yellow petals into small non-light reflecting structures such as sepals. Such an organ conversion is preferable over the removal of the petals to avoid interfering with insect pollination. Pierre et al. (1996) have shown that honeybees, the main

M. Byzova · C. Verduyn · D. De Brouwer  
M. De Block (✉)  
Bayer BioScience N.V., Jozef Plateastraat 22,  
9000 Gent, Belgium  
E-mail: marc.deblock@bayercropscience.com  
Fax: + 32-9-2240694

pollinators on oilseed rape, do not crawl over the anthers and stigma of apetalous flowers as they do in petalous ones but often insert their tongues between the sepals to collect the nectar. In this way pollination might be reduced, resulting in a lower seed set.

The molecular mechanisms governing floral organ identity are well understood. According to the "A-E" model, the organ identity of each floral whorl is determined by a unique combination of four organ identity activities, called A, B, C and E (Weigel and Meyerowitz 1994; Pelaz et al. 2000; Jack 2001; Theissen 2001; Fig. 1). Expression of the (A)-type genes specifies sepal formation. The combination of (A + B + E) activities spec-

ifies the formation of petals, while combined (B + C + E) functions specify stamen formation. Expression of the (C + E)-type genes determines the development of carpels (Fig. 1). All types of organ identity genes have been cloned from *Arabidopsis*. An example of the A-type gene is *API* (Mandel et al. 1992). The B-type genes are *AP3* (Jack et al. 1992) and *PI* (Goto and Meyerowitz 1994), and the C-type gene is *AGAMOUS* (*AG*) (Yanofsky et al. 1990). The E-function is provided by three *SEPALLATA* genes (Pelaz et al. 2000). All these genes are transcription factors belonging to the MADS-box gene family.

In this paper, silencing of the B-type MADS-box genes in a second-whorl-specific manner was obtained in both *Arabidopsis* and *B. napus* flowers by expressing a *B. napus* B-type gene hpRNA construct under control of an *Arabidopsis* A-type MADS-box gene promoter (Fig. 1). In this way, *Arabidopsis* lines with double sepaloid flowers and *B. napus* lines with flowers in which petals are converted into sepaloid petals were generated. The novel flower phenotypes were stable and heritable in both species.

## Materials and methods

### Plant material

*Arabidopsis thaliana* (L.) Heynh. ecotype C24, kindly provided by Dr. M. Van Lijsebettens (VIB, Gent, Belgium), and the double haploid *Brassica napus* L. line cv. Simon (Bayer BioScience N.V., Gent, Belgium) were used in this study.

### Plasmid construction

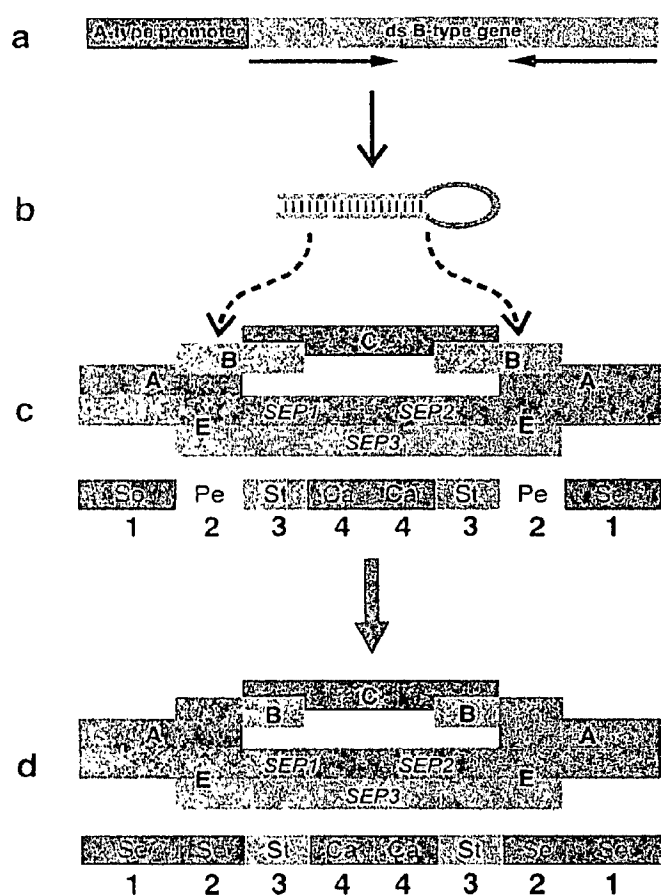
The 3'-coding regions of the *BAP3* and *BPI* genes were cloned by means of RT-PCR performed on total RNA isolated from *B. napus* flower buds. RT-PCR was performed according to the protocol of the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). *AP3* cDNA-specific primers:

- 5'-CGCACTCAGATTAAGCAGAGGC-3' and
- 5'-GGAAGGTAATGATGTCAGAGGC-3'

and *PI* cDNA-specific primers:

- 5'-GGGAGAAGATATACAGTCTCTCAAC-3' and
- 5'-GAATCGGTTGCACTCTATATCC-3'

were chosen based on the published sequences (Jack et al. 1992, GenBank Accession D30807; Goto and Meyerowitz 1994, GenBank Accession M86337). In the pAPI::hpBAP3 construct, one of the *BAP3*-specific DNA fragments, 380-bp in length, was cloned as an inverted repeat with the  $\beta$ -glucuronidase (GUS) fragment containing nucleotides 744–975 as a spacer. In the pAPI::hpBPI construct, one of the *BPI*-specific fragments, 255-bp in length, was cloned as an inverted repeat with the intron IV2 from the potato light-inducible tissue-specific gene ST-LS1, 251-bp in length, as a spacer (Vancanneyt et al. 1990). In the pAPI::hpBAP3 and pAPI::hpBPI constructs, gene-specific structures were driven by a 1,182-bp fragment of the *API* promoter. The fragment of the *API* promoter (–1182 to +1) was cloned by means of PCR from pKY65 plasmid kindly provided by Martin Yanofsky. In p $\Delta$ AP3-AP1::hpBPI fragments of the *AP3* promoter, containing nucleotides –727 to –556 and –224 to –1 were cloned by PCR based on



**Fig. 1a–d** Experimental strategy. **a** Representation of the basic construct used in this study. A DNA fragment of a 3'-coding region of a B-type MADS-box gene (yellow box) was sub-cloned as an inverted repeat (directions are indicated by arrows) with a part of the GUS gene or the intron IV2 from gene ST-LS1 (Vancanneyt et al. 1990) as a spacer (blue box). The constructs were driven by an A-type MADS-box gene promoter (green box). **b** Transcripts produced by the construct are predicted to form a hairpin structure. **c** Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in wild-type plants. **d** Domains of the A, B, C and E functions and the corresponding organ identities in floral whorls in transgenic plants. In transgenic plants, down-regulation of the B-type function in the second whorl only leads to development of sepals instead of petals. Numbers indicate whorls. *Se* sepals, *Pe* petals, *St* stamens, *Ca* carpels

the published sequence (Irish and Yamamoto 1995, GenBank Accession U30729) and linked to the 5'-end of the *AP1* promoter. Plasmid constructs were introduced into *Agrobacterium tumefaciens* strain C58C1rif by electroporation.

#### Plant transformation

The transformations of *A. thaliana* and *B. napus* were essentially done as described by Valvekens et al. (1992) and De Block et al. (1989), respectively.

#### Cytology

The embedding was done in Historesin as advised by the manufacturer (Leica, Heidelberg, Germany). Sections 5  $\mu$ m thick were stained with 0.05% toluidine blue.

#### In situ hybridization

Embedding in methacrylate, sectioning, and the removal of the plastic were essentially done as described by Baskin et al. (1992). The in situ hybridizations on 7- $\mu$ m sections were essentially done as described by De Block and De Brouwer (1993).

#### Microscopy

Sections were examined with an Axioplan (Zeiss, Jena, Germany) microscope equipped with Normaski differential interference contrast.

#### Spectrophotometric determination of chlorophyll

The total chlorophyll (*a* + *b*) content was measured as described by Bruisma (1963).

## Results

### General strategy: silencing the B-type MADS-box genes in a second floral whorl-specific manner

To convert petals into sepals without interfering with anther development, the strategy outlined in Fig. 1 was used. Following the A–E flower development model it is expected that silencing of a B-type MADS-box gene, *AP3* or *PI*, in the second whorl will redirect the development of petals into sepals. This could be obtained by expressing in the second, but not in the third whorl self-complementary 'hairpin' RNA (hpRNA) constructs containing *AP3*- and/or *PI*-specific sequences. Down-regulation of the B-type MADS-box genes in the third whorl has to be avoided to maintain normal male fertility. For this purpose an A-type promoter driving the expression of the hpRNA construct could be used.

Starting from the *PI* and *AP3* sequences (Jack et al. 1992; Goto and Meyerowitz 1994), we identified in the amphidiploid *B. napus* five *AP3*-like (*BAP3*) and three *PI*-like (*BPI*) genes that were actively expressed during flower development (data not shown). Fragments of the 3'-coding region of the *BAP3* and *BPI* genes were isolated. The nucleotide sequence similarity between

members of the same B-type MADS-box gene subfamily turned out to be on average 95%. Each *B. napus* gene subfamily shared with its unique *Arabidopsis* counterpart about 91% sequence similarity, containing multiple blocks of more than 20 bases of perfect homology. This high sequence similarity should be sufficient to silence the target genes in both *Arabidopsis* and *B. napus* by using the same hpRNA constructs (Helliwell and Waterhouse 2003). The feasibility of the strategy to convert petals into sepals by silencing the B-type MADS-box genes only in the second floral whorl was first evaluated in the model plant *Arabidopsis thaliana*.

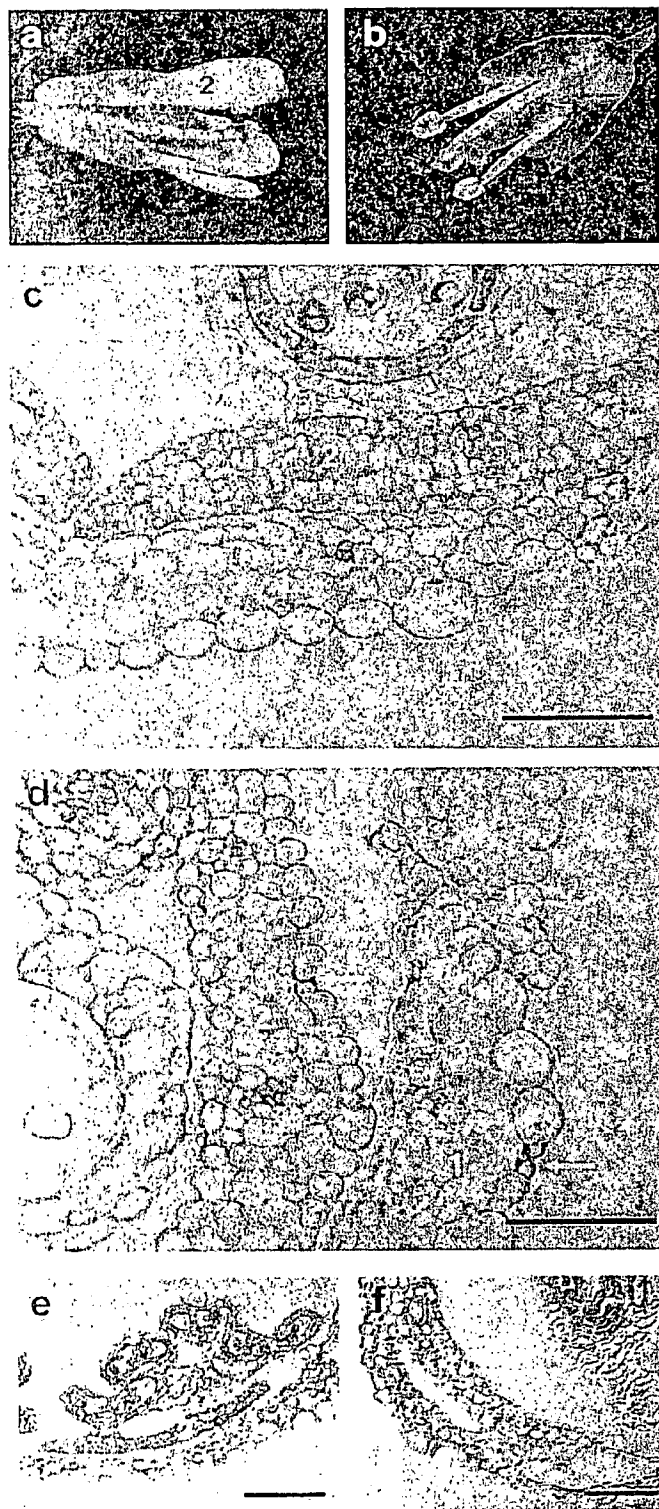
### Generation of *Arabidopsis* transgenic lines with male fertile double sepaloid flowers

To make constructs that produce hpRNA B-type MADS-box gene transcript, the 3'-coding regions of one *BAP3* and one *BPI* gene, were subcloned as an inverted repeat (see Materials and methods). Both hpBAP3 and hpBPI gene-specific sequences were driven by a 1.1-kb promoter fragment of the *Arabidopsis AP1* gene. The resulting pAP1::hpBAP3 and pAP1::hpBPI constructs were introduced separately into *Arabidopsis*.

A total of 125 pAP1::hpBAP3 and 56 pAP1::hpBPI transgenic lines was generated. All the plants were normal in terms of vegetative growth while they had morphological changes in flower organs. 16.9% of the pAP1::hpBPI and 5.6% of the pAP1::hpBAP3 lines exhibited the desirable double sepaloid phenotype (Fig. 2b). Instead of petals, sepals developed in the second floral whorl, indistinguishable from those of the first whorl except for their slightly smaller size. Despite their transformation, these organs developed in the positions and on a time course characteristics of petals. Some other pAP1::hpBAP3 *T*<sub>0</sub> plants had a range of phenotypes related to the severity of homeotic transformations observed in petal and stamen development. 10.4% of the pAP1::hpBAP3 lines produced flowers with short white petals and 20% of the lines had homeotic aberrations in stamens ranging from weak carpelloidy to complete transformation of stamens into carpels (Table 1). In contrast to the pAP1::hpBAP3 lines, no aberrations in the third floral whorl were observed in the pAP1::hpBPI transgenic plants (Table 1).

Microscopic analysis of cross-sections of mature pAP1::hpBPI double sepaloid flowers revealed that the mesophyll cells of the second-whorl organs were sepaloid in nature, as indicated by the presence of chloroplasts and their larger size than those normally found in wild-type petals. The abaxial epidermis was like that of sepals, consisting of stomata and irregularly shaped cells (Fig. 2d). The same results were obtained for pAP1::hpBAP3 double sepaloid flowers (data not shown).

To confirm that the double sepaloid phenotype of *Arabidopsis* transgenic plants was caused by depletion of expression of endogenous B-type homeotic genes in the



second whorl, the *PI* mRNA expression pattern in pAP1::hpBPI was examined by in situ hybridization. In wild-type *Arabidopsis* flowers, *PI* mRNA is detected

Fig. 2a–f Analysis of the double sepaloid pAP1::hpBPI *Arabidopsis thaliana* flowers. a C24 *Arabidopsis* wild-type mature flower. b Mature transgenic flower. Second-whorl organs are sepals (arrow) that are slightly smaller than the true sepals. c, d Cytological transverse sections taken approximately in the middle of anthers of flower buds at stage 12. c Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of the second-whorl petals are smaller than those of sepals developed in the first whorl. Abaxial epidermal cells of petals are regular in shape. d Cellular morphology of first- and second-whorl organs of a transgenic flower. Mesophyll and epidermal cells of the second-whorl organs are slightly smaller in size than cells of the first-whorl sepals. The shape of the cells of the second-whorl sepals is similar to those of the first-whorl sepals. Stomata (arrows) are present in the abaxial epidermis of the second-whorl organs as in normal first-whorl sepals. e, f In situ analysis of *PI* expression in transverse sections of wild-type and transgenic flowers. The hybridisation signal is confined to the second- and third-whorl organs in wild-type flowers (e). In transgenic flowers (f) *PI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 50  $\mu$ m (c, d, f), 100  $\mu$ m (e)

from stage 3 (Smyth et al. 1990) in second- third- and fourth-whorl primordia. In the second and third whorls it persists until anthesis (Goto and Meyerowitz 1994). In pAP1::hpBPI double sepaloid flowers the *PI* transcript was not detectable in the second-whorl primordia at any of the stages examined (Fig. 2f). Expression of *PI* in developing stamens was similar to that observed in the wild type.

As it has been shown that expression of both *PI* and *AP3* genes is reduced when either the *PI* or *AP3* gene is mutated (Jack et al. 1992; Goto and Meyerowitz 1994), we anticipated that inhibition of expression of one of the B-type MADS-box genes in a tissue where both genes are active would lead to reduction of expression of the counterpart gene in the same manner. To verify this hypothesis, in situ hybridization of the pAP1::hpBPI flowers using the *BAP3*-specific fragment as a probe was performed. As was predicted, *AP3* RNA was not detected in the second whorl of developing organs. However, no reduction in the level of the *AP3* mRNA was observed in stamens (data not shown).

Heritability and stability of the double sepaloid trait was tested by self-pollination. The trait was heritable and in the case of pAP1::hpBPI stable through the T<sub>1</sub> and T<sub>2</sub> generations. In the case of pAP1::hpBAP3 some T<sub>1</sub> and T<sub>2</sub> lines produced flowers with homeotic aberrations in stamens, as previously observed in the T<sub>0</sub> plants.

In *B. napus*, silencing of B-type MADS-box genes in the second whorl results in the transformation of petals into sepaloid petals

To evaluate whether the expression of the pAP1::hpBAP3 and pAP1::hpBPI genes would also result in a double sepaloid phenotype in *B. napus*, 48 and 53 transgenic lines, respectively, were generated.

All the pAP1::hpBAP3 lines had wild-type flowers. Among the pAP1::hpBPI transgenic lines, 22.6%

**Table 1** Phenotypic analysis of *T<sub>0</sub>* *Arabidopsis thaliana* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)		
		Double sepaloid Fertile	Double sepaloid Partially male sterile <sup>a</sup>	Short petals Fertile
pAPI::hpBPI	56	16.9	< 2	18.9
pAPI::hpBAP3	125	5.6	20	10.4

<sup>a</sup>Range of aberrations in stamens from mild to complete conversion of stamens into carpels

exhibited an apetalous or partially apetalous phenotype characterised by the appearance of flowers without petals or bearing 1, 2 or 3 petals only (Table 2). Frequently, the petals were significantly smaller and narrower than those from wild type (data not shown). However, this phenotype was unstable and not heritable.

The absence of the double sepaloid phenotype in transgenic *B. napus* lines with the same constructs used in *Arabidopsis* could be due to an inability of the *Arabidopsis* *API* promoter to direct transcription of adequate amounts of double-stranded transcripts necessary to trigger silencing of all target *BAP3* or *BPI* genes expressed in rapeseed flowers. Starting from this hypothesis, a new construct was generated that could produce higher amounts of hpRNA. Because the pAPI::hpBAP3 *B. napus* transgenic plants did not exhibit any phenotypes different from those of wild-type plants, and in *Arabidopsis* the most stable double sepaloid flower phenotype was obtained with the hpBPI construct, we continued only with the hpRNA *BPI* gene.

To enhance the level of expression of hpBPI specifically in the second whorl, an *Arabidopsis* modified *AP3* regulatory fragment was added to the *API* promoter.

Discrete *cis*-acting elements regulating spatial and temporal expression of the *Arabidopsis* *AP3* gene have been identified (Hill et al. 1998; Tilly et al. 1998). Based on these data the positive regulator of the *AP3* expression during the early stages of flower development was combined with the petal-specific regulatory region (see Materials and methods). The modified *AP3* promoter was introduced in the pAPI::hpBPI construct directly upstream of the *API* sequence. This pΔAP3-*API*::hpBPI construct was transformed into *B. napus*.

Of the 125 primary transformants, 11.2% produced flowers with aberrant second-whorl organs. Of these 11.2% lines, half (5.6%) produced flowers in which petals were converted into sepaloid petals (Fig. 3a, Table 2). These organs were yellowish-green, indicating the presence of chloroplasts in their cells that is characteristic of wild-type sepals. The size of the sepaloid petals was comparable to the size of true sepals. These organs

were narrow and almost strap-like in shape, like sepals, but had a small lamina and base, characteristic of a petal. In addition the lamina portion was wrinkled (Fig. 3a).

The aberrant *B. napus* flowers with sepaloid petals were analysed microscopically to verify the identity of tissues in the second-whorl organs. As shown in Fig. 3c the size and the shape of epidermal and mesophyll cells of these organs were indistinguishable from the first-whorl sepals. Moreover, the mesophyll cells of the sepaloid petals contained a large number of chloroplasts (Fig. 3d).

In addition, spectrophotometric analysis of chlorophyll fluorescence, which was done on the first and the second floral organs of transgenic plants, revealed that chlorophyll content in the sepaloid petals is only 30% less than in the true wild-type sepals (data not shown).

In situ hybridization of flower sections with a *BPI*-specific probe confirmed the absence of a detectable level of *BPI* gene expression in the second whorl of the transgenic flowers, indicating that the complete *BPI* gene family was down-regulated (Fig. 3e).

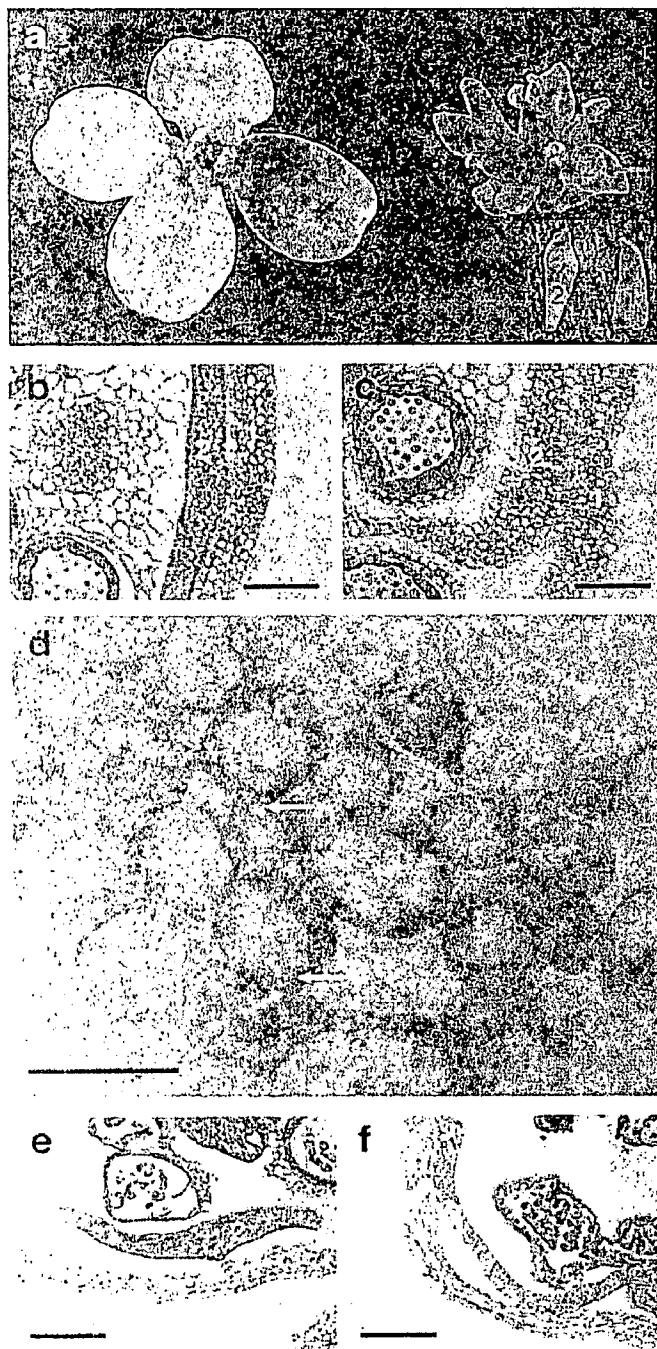
The other half of the 11.2% transgenic pΔAP3-*API*::hpBPI lines exhibited partial apetalous and apetalous phenotypes similar to those observed in pAPI::hpBPI transgenic plants (Table 2).

The flower phenotype with sepaloid petals is a stable trait in *B. napus* transgenic plants

The stability of transformation of petals to sepaloid petals in *B. napus* was tested for six lines, of which the original *T<sub>0</sub>* plants had flowers with sepaloid petals and contained only one copy the pΔAP3-*API*::hpBPI transgene. The *T<sub>0</sub>* plants were first maintained by selfing. The transgenic plants of these *T<sub>1</sub>* generations had flowers with sepaloid petals while the azygous segregants had normal wild-type flowers. For each line ten transgenic plants of the *T<sub>1</sub>* generation were backcrossed with the original non-transgenic double haploid *B. napus* line cv.

**Table 2** Phenotypic analysis of *T<sub>0</sub>* *Brassica napus* plants

Transformed constructs	Total number of transgenic lines	Plants with mutant phenotype (%)	
		Sepaloid petals	Apetalous/partially apetalous
pAPI::hpBAP3	48	0	0
pAPI::hpBPI	53	0	22.6
pΔAP3- <i>API</i> ::hpBPI	125	5.6	5.6



Simon. Depending on whether the  $T_1$  plant used was homo- or heterozygous for the transgene, all or 50% of the  $F_1$  plants, respectively, had sepaloid petals in their flowers. A second backcross was done with 15 plants of each line. As expected, in the  $F_2$  generations there was a 1:1 segregation of wild-type plants and plants with sepaloid petals. The flower phenotype of the transgenic  $F_2$  plants was identical to those of the  $T_0$ ,  $T_1$  and  $F_1$  transgenic plants.

**Fig. 3a–e** Analysis of the pΔAP3-AP1::hpBPI *B. napus* flowers. **a** Morphological features of *Brassica napus* flowers: mature wild-type flower (left), mature flower of a transgenic plant (right). The second-whorl organs of a transgenic flower are yellowish-green sepaloid petals (arrow). The size of these organs is similar to sepals developed in the first whorl, but the lamina-base structure can still be distinguished (for comparison see the detached organs in the bottom right corner: the second-whorl organ (left), the first-whorl organ (right) of a transgenic flower). **b–d** Cytological transverse sections taken approximately in the middle of anthers at the early yellow bud stage (Smith and Scarisbrick 1990). **b** Cellular morphology of first- and second-whorl organs of a wild-type flower. Mesophyll cells of petals are smaller than those of sepals. Epidermal cells of petals are regular in shape. **c** Cellular morphology of the first- and second-whorl organs of a transgenic plant. The shape and the size of mesophyll and epidermal cells of the second-whorl organs are similar to those of the first-whorl sepals. **d** Cytology of a sepaloid petal showing the presence of chloroplasts (two examples indicated by arrows) in the mesophyll cells. **e, f** In situ analysis of *BPI* expression on transverse sections of wild-type and transgenic flowers. The hybridization signal is confined to the second- and the third-whorl organs in wild-type flowers (**e**). In transgenic flowers (**f**) *BPI* expression is detected in the third-whorl organs only. Numbers indicate whorls. Bars = 20  $\mu$ m (**d**), 100  $\mu$ m (**b, c**), 200  $\mu$ m (**e, f**)

## Discussion

The hpRNA-mediated gene silencing technology has been proven to be a very efficient tool for gene discovery and functional genomics in diverse organisms such as fungi (Pickford et al. 2002), nematodes (Bargmann 2001), and animals (Harborth et al. 2001). In plants this technology has been used successfully to generate virus resistance (Waterhouse et al. 1998) as well as to obtain consistent and profound inhibition of the expression of transgenes and endogenous genes (Levin et al. 2000; Smith et al. 2000; Wesley et al. 2001; Liu et al. 2002).

Chuang and Meyerowitz (2000) demonstrated that the hpRNA-mediated silencing technology could be used to interfere with flower development. A range of aberrant flower phenotypes was obtained by down-regulating the floral organ genes *AGAMOUS*, *CLAVATA3*, *APET-ALAI*, and *PERIANTHIA* using hpRNA constructs driven by the constitutive 35S and nopaline synthase promoters. Recently, it has been shown that the hpRNA-mediated silencing technique can be used to silence genes in an organ-specific way. The fatty acid composition of *Arabidopsis* and cotton seeds was modified by down-regulating the seed expression of two fatty acid desaturase genes using hpRNA constructs driven by seed-specific promoters (Liu et al. 2002; Stoutjesdijk et al. 2002).

In this article we present for the first time to our knowledge implementation of the hpRNA-mediated technology to silence a multigene family in a floral whorl-specific manner. Silencing of the B-type MADS-box genes that are present in single copy in *Arabidopsis* but are present in multiple copies in *B. napus* causes complete transformation of petals to sepals in *Arabidopsis* and partial transformation in *B. napus*. This flower phenotype is stable and heritable in both species.

In *Arabidopsis*, unlike the silencing of the *PI* gene, silencing of the *AP3* gene results in homeotic aberrations in anthers in 20% of the cases. This implies that in these lines partial silencing of the *AP3* gene also occurs in developing stamens. These results can be attributed to two possibilities. First, in wild-type *Arabidopsis* flowers *API* is expressed during early floral stages throughout all four whorls and is down-regulated in whorls 3 and 4 by the *AG* gene during stage 3, persisting in whorls 1 and 2 only (Mandel et al. 1992; Bowman et al. 1993). However, in contrast to the endogenous promoter, the smaller *API* promoter fragment we used might have some activity in the central whorls after stage 3 as proposed by Yun et al. (2002). The activity of the pAPI::hpBAP3 gene might have led to down-regulation of *AP3* in the third floral whorl. Alternatively, an aberrant stamen development in the pAPI::hpBAP3 transgenic plants might be the result of the spreading of a silencing signal between floral whorls.

Both hypotheses imply that a certain amount of dsRNA of the *AP3* gene present in the third whorl of transgenic flowers is sufficient to trigger silencing of *AP3*. This is not the case for the *PI* gene, for which the down-regulation did not result in aberrant anther phenotype. *PI* and *AP3* are both expressed in developing petals and stamens. However *PI* expression levels are similar in both whorls, whereas *AP3* expression is lower in developing stamens than in petals (Zhou et al. 2002). It may be that for this reason a lower threshold concentration of hpRNA is required in stamens to provoke a partial inhibition of the *AP3* gene expression.

Although systemic spreading of silencing may be a concern for implementation of the hpRNA-mediated silencing technology in tissue-specific applications in plants (Wang and Waterhouse 2002), the stability of the aberrant flower phenotype throughout development of our transgenic plants indicates that at least in the case of the B-type MADS-box genes there is no significant spreading of silencing between the meristems of adjacent floral organs.

Another phenomenon that might limit application of the hpRNA gene silencing technique is spreading of RNA targeting. During this process spreading of the RNA silencing signal occurs from the initial target sequence into the adjacent 5' and 3' regions (Jones et al. 1999; Vaistij et al. 2002). This may result in the participation of the entire transcribed region of the target gene in the RNA silencing process. As a consequence, expression of other homologous genes can be inhibited. Based on this hypothesis and the fact that different types of MADS-box genes share a high percentage of homology at the MADS-box regions (Purugganan et al. 1995), target-site spreading along the *AP3* or *PI* transcribed sequences would lead to silencing of not only *AP3* and *PI* but also of other MADS-box genes that are expressed in the developing second-whorl organs. In this case petals will be converted not only into sepals but also into organs with staminoid and/or carpeloid and/or other aberrant

structures. The absence of such phenotypes in our transgenic plants suggests that silencing of B-type MADS-box genes was not associated with the spreading of RNA targeting. The absence of the target-site spreading process was also observed by Vaistij et al. (2002) for the ribulose-1,5-bisphosphate carboxylase/oxygenase and phytoene desaturase genes. These results demonstrate that the hpRNA-mediated gene silencing technology can be applied not only to silence all genes of a multigene family but also to silence specifically a single member of a subfamily or even of a multigene family.

*B. napus* plants transformed with the improved pΔAP3-API::hpBPI construct have small yellowish-green sepaloid petals in the second whorl. Although mesophyll and epidermal cells of these sepaloid petals are sepaloid in morphology, the light-yellow colour suggests that some petal-specific biochemical pathways are still active in the cells of these organs. In addition, the small lamina and base of these organs are petal characteristics. It might be that undetectable levels of *BPI* transcripts are still sufficient for maintenance of some petaloid features.

Recently, in *Arabidopsis* an alternative approach was used to interfere with the expression of *AP3* in a second-whorl-specific manner (Guan et al. 2002). A zinc finger protein designed to bind to a region upstream of *AP3* was fused to the human transcriptional repression domain of mSIN3. When the *API* promoter was used to drive the expression of this artificial zinc finger transcription factor, flowers were obtained that were partially apetalous or that contained some sepaloid petals. Although the use of synthetic transcription factors is a promising approach to interfere with gene regulation, high expression levels of these transcription factors are probably needed to obtain a full phenotype by gene repression. Due to technical limitations the use of such artificial transcription factors is less feasible when multiple genes with redundant function, like the B-type MADS-box genes in *B. napus*, have to be repressed.

Theoretically, in *Arabidopsis* a double sepaloid flower phenotype may also be obtained by silencing the *SEP-ALLATA* genes in the second whorl (Fig. 1). However, due to the redundant function of the *SEPALLATA* genes, all three genes would have to be silenced together (Pelaz et al. 2000).

In conclusion, *Arabidopsis* and *B. napus* lines with a flower phenotype that is, respectively, double sepaloid or has sepaloid petals, and that is male fertile and stable in subsequent generations can be obtained by a hpRNA-mediated gene silencing of the *PISTILLATA* gene exclusively in the second floral whorl. Further physiological studies of *B. napus* transgenic lines will allow quantification of the effect of the flower architecture with sepaloid petals on the distribution of PAR and on other important agronomic features such as pollination and overall seed yield.

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# Exhibit 21

Attorney's Docket No. 1021565-000060

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Ming-Bo Wang et al.	)	Group Art Unit: 1635
	)	
Application No.: 09/287,632	)	Examiner: JANE J ZARA
	)	
Filed: April 7, 1999	)	Confirmation No.: 6526
	)	
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED PHENOTYPES	)	
	)	
	)	
	)	

### DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Peter Robert Schofield of the Prince of Wales Medical Research Institute, Sydney, New South Wales, 2010, Australia, declare as follows:

1. I am currently the Executive Director and CEO of the Prince of Wales Medical Research Institute. I also hold an appointment as a Professor of Medicine at the University of New South Wales.
2. I graduated from the University of Sydney with the degree of Bachelor of Agricultural Science with Honours in 1982. In 1985, I received my PhD in genetics from the Australian National University. In 1998, I was awarded the degree of Doctor of Science by the University of New South Wales. I have published over one hundred and ninety scientific papers in the fields of genetics, molecular biology and neuroscience. I have given presentations at many local and international conferences and serve on several government committees in the healthcare/pharmaceuticals area. A brief form of my CV is attached as Exhibit 1.
3. This declaration relates to an examination by the United States Patent and Trademark Office (USPTO) to United States Patent Application No. 09/287,632 in the name of Commonwealth Scientific and Industrial Research Organisation ("CSIRO").

4. In view of my qualifications and experience in molecular biology, especially my knowledge of gene cloning and gene structure, I believe I am qualified to comment on the technical aspects of the United States Patent Application No. 09/287,632 (hereinafter "the '632 application").
5. I have read and understood the following documents which are relevant to the present examination:
  - a) Complete specification of PCT Patent Application No. WO 99/53050, which is equivalent to the '632 application;
  - b) An Office Action from the USPTO dated February 8, 2007 in connection with the '632 application;
  - c) The pending claims of the '632 application.
6. I have been requested by the CSIRO to provide my comments in relation to the statements made by the USPTO examiner in the Office Action, specifically with regard to claims 85, 91, 106, 107 and 108, which recite the inclusion of an intron.
7. Unless stated to the contrary, when I express an opinion in this Declaration, I am expressing the view that I believe a person of ordinary skill in the field of the '632 application would have held as of 8 April 1998, the priority date of the application.

#### **My Opinion**

8. Example 6 of the specification describes the use of an intron (*Flaveria trinervia* pyruvate orthophosphate dikinase intron 2) in a DNA region encoding sense and antisense sequences. Tobacco plants transformed with the constructs were subsequently challenged with PVY. The results presented in Table 8 (page 47) show that the inclusion of an intron (in either the sense or antisense orientation) resulted in 22 or 21 of the 24 independent transgenic plants being immune to PVY challenge, respectively. This surprising result represented the highest percentage of plant resistance to disease reported in the entire specification, indicating that inclusion of an intron provided a highly desirable feature in construct design and treatment efficacy.
9. This is further explained in the specification (page 23) in which it is stated "In fact, the inventors have unexpectedly found that inclusion of an intron sequence in the chimeric DNA genes encoding an RNA molecule comprising the hairpin RNA, preferably in the spacer region, and preferably in the sense orientation, enhances the efficiency of reduction of expression of the target

nucleic acid. The enhancement in efficiency may be expressed as an increase in the frequency of plants wherein silencing occurs, or as an increase in the level of reduction of the phenotypic trait."

10. Example 1 of the specification (page 36) also makes reference to introns. In this case it states "a castor bean catalase intron (Ohta et al., 1990) as modified by Wang et al. (1997) ("intron")." Subsequently it is stated (page 37) "In addition, T-DNA vectors were constructed to evaluate the influence of a presence of an intron sequence in the chimeric genes encoding CoP constructs."
11. The results of these experiments are reported in Example 1 and Table 2. It states (page 39) "Supertransformation with GUSd in a sense or antisense orientation, with or without an intron or an early stop codon, showed some degree of reduction (in about 25% of the analysed calli) of the endogenous GUS activity".
12. Thus, the specification makes reference to introns generally, as discovered and described by Sharp and Roberts, and then proceeds to demonstrate the effects of using two different intron sequences, supporting the view that the inclusion of an intron (any intron) will confer the desirable features of the invention.
13. A person of ordinary skill in the field, would have understood that introns are part of the transcribed DNA sequence of a gene that is non-coding or intervening, and are removed from the heterogeneous nuclear RNA (hnRNA or pre-mRNA) transcript of the gene by the spliceosome complex. The spliced hnRNA becomes the mRNA. Most, but not all, eukaryotic genes contain introns, as do mitochondrial and chloroplast genes.
14. Introns were discovered in 1977 by Phillip Sharp and Richard Roberts. Their discovery altered the previous view that genes were continuous stretches of DNA that served as direct templates for mRNA molecules, which form the templates for protein synthesis. This discovery was recognized by the award of the Nobel Prize for Physiology or Medicine in 1993. The press release, issued by the Nobel Assembly, about this work and its significance is notable because it refers to introns as a class, and does not make specific distinctions about any particular intron. The press release is attached hereto as Exhibit 2.
15. The general nature of this discovery, and its translation to all of eukaryotic cell biology was such that it was featured in both the most highly rated scientific journals such as *Science* and in the

more generally accessible journals such as *Scientific American*. For example, Francis Crick stated in a review in *Science* on "Split genes and RNA splicing" (*Science* 204: 264-271, 1979) that "A number of genes in higher organisms and in their viruses appear to be split. That is, they have "nonsense" stretches of DNA interspersed within the sense DNA. The cell produces a full RNA transcript of this DNA, nonsense and all, and then appears to splice out the nonsense sequences before sending the RNA to the cytoplasm. In this article what is known about these intervening sequences and about the processing of the RNA is outlined. Also discussed is their possible use and how they might have arisen in evolution." Similarly, an article by Pierre Chambon titled "Split Genes" was published in 1981 describing the discovery and characterization of introns (*Scientific American* 244, 60-71).

16. This view about the generality of introns was supported by the research that I undertook, the journal articles that I read and the work conducted by my colleagues in the same laboratory from the commencement of my PhD studies in 1982 and further from the commencement of my postdoctoral studies in 1985.
17. Specific examples of work involving the identification of introns and confirming that all introns conform to a common design was undertaken in my laboratory by my colleagues. For example, in 1982, Dr Robert Richards published an article in the journal *Nature* in which they reported the complete nucleotide sequence of two members of the human metallothionein gene family. They were able to deduce that one gene was a functional metallothionein-II gene, while the other was a pseudogene, lacking introns, but terminating in a poly(A) tail. (Karin, M and Richards, RI. (1982) Human metallothionein genes--primary structure of the metallothionein-II gene and a related processed gene. *Nature* 299: 797-802).
18. In another study undertaken my fellow PhD student, Anthony Mason, he characterized and published an article in the journal *Nature* describing the structure, including intron-exon structure, of a mouse genomic clone containing a complete kallikrein gene (mGK-1) and the 3' end of another (mGK-2). (Mason, AJ, Evans, BA, Cox DR, Shine, J and Richards RI. (1983) Structure of mouse kallikrein gene family suggests a role in specific processing of biologically active peptides. *Nature* 303: 300-307).
19. Other research in my laboratory concerned the cloning of the leghaemoglobin gene from the soybean. Both Marcker's and Verma's laboratories reported the primary gene structures of structures of soybean leghemoglobin genes. It will be noted that the term 'primary structure'

means the genomic DNA structure, from which, by analysis of the mRNA structure, as deduced by analysis of cDNA sequences, it is possible to simply deduce the intron-exon structure of the gene. Hyldig-Nielsen et al. in their paper 'The primary structures of two leghemoglobin genes from soybean' (*Nucleic Acids Res* 10: 689-701, 1982) presented "the complete nucleotide sequences of two leghemoglobin genes isolated from soybean DNA. Both genes contain three intervening sequences which interrupt the two coding sequences in identical positions." They concluded that "the general DNA sequence organization of these plant genes is similar to that of other eukaryotic genes."

20. Wiborg et al. in their paper 'The nucleotide sequences of two leghemoglobin genes from soybean' (*Nucleic Acids Res* 11: 3487-94, 1982) stated "We present the complete nucleotide sequences of two leghemoglobin genes isolated from soybean DNA. Both genes contain three intervening sequences in identical positions."
21. Brisson and Verma in their paper "Soybean leghemoglobin gene family: normal, pseudo, and truncated genes" (*Proc Natl Acad Sci USA* 79: 4055-4059, 1982) stated "Leghemoglobin (Lb) genes in soybean represent a small family of closely related genes. Three Lb sequences isolated from a genomic library were analyzed at the nucleotide sequence level. A Lb gene present on an 11.5-kilobase (kb) EcoRI genomic fragment spans approximately 1,200 nucleotides and is interrupted at amino acid positions 32 to 33, 68 to 69, and 103 to 104. The intervening sequences, as well as the 5' and 3' flanking regions of this gene, contain the consensus sequences found in other eukaryotic genes."
22. At the same time Stephen Mount published a paper entitled "A catalogue of splice junction sequences" (*Nucleic Acids Res* 10: 459-472, 1982) which has attracted numerous citations for the consensus sequences that define intron-exon splice boundaries. The paper's abstract states "Splice junction sequences from a large number of nuclear and viral genes encoding protein have been collected. The sequence CAAG/GTAGAGT was found to be a consensus of 139 exon-intron boundaries (or donor sequences) and (TC)nNCTAG/G was found to be a consensus of 130 intron-exon boundaries (or acceptor sequences). The possible role of splice junction sequences as signals for processing is discussed."
23. The work by Mount defined the GT-AG sequences as forming the boundaries of all eukaryotic introns. Because of this, it has been possible to define and use introns irrespective of location in a gene or the organism from which the gene was isolated.

24. The Office Action states (page 4) "The specification, claims and the art do not adequately describe the distinguishing features or attributes concisely shared by the members of the broad genus comprising DNA constructs whereby any intronic sequence is inserted anywhere in the chimeric DNA, and whereby the DNA construct provides for the function claimed, of generating a gene silencing construct that reduces phenotypic expression of any nucleic acid of interest in any plant and in any eukaryotic cell." Further the examiner states "The specification fails to teach or adequately describe a representative number of species in the genus such that the common attributes or characteristics concisely identifying members of the proposed genus are exemplified (e.g. the myriad of sequences encompassed by the genus intron, or intronic sequences is vast, and further whereby any intronic sequence is inserted anywhere within the DNA construct and a DNA chimeric construct generates a gene silencing construct which reduces the phenotypic expression of any nucleic acid of interest in any eukaryotic cell). And because the genus claimed is so highly variant, the description provide is insufficient whereby a representative number of chimeric constructs provide for the functions claimed, of reducing the phenotypic expression of any nucleic acid of interest in any eukaryotic cell or plant. One of skill in the art would reasonably conclude that the disclosure, at the time of filing, fails to provide a representative number of species to describe the broad genus claimed. Thus, Applicant was not in possession of the claimed genus."
25. As noted above, the specification teaches two intron sequences, not one as stated in the Office Action (page 4).
26. With regard to the state of the art, by the early 1980s it was clear that the broad genus 'intron' could be easily and reliably defined by comparison of genomic DNA and cDNA (or mRNA) sequences, and that there were well defined elements that allowed the categorical definition of introns.
27. That the inclusion of an intron significantly enhances the effectiveness of the construct in reducing target gene expression is abundantly clear from the specification of the Application.
28. The Office Action also refers to statements in Smith et al. *Nature* 407: 319-320, 2000. For example, the examiner quotes Smith who state "How does the presence of this intron enhance silencing efficiency? The process of intron excision from the construct by the spliceosome might help align the complementary arms of the hairpin in an environment favouring RNA hybridization, promoting the formation of a duplex. Alternatively, splicing may transiently

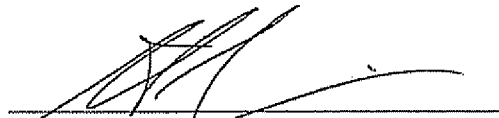


increase the amount of hairpin RNA by facilitating, or retarding, the hairpin's passage from the nucleus, or by creating a smaller, less nuclease-sensitive loop."

29. Ultimately, the mechanism by which the presence of an intron results in the enhanced efficiency of target gene silencing is not relevant to what is being claimed in the current claims of the '632 application. A person of ordinary skill in the field of the application would have understood from reading its specification that the presence of an intron (generally any intron) will enhance the target gene silencing. This is supported by the examples in the specification in the light of the prior art literature which clearly specifies and defines the broad genus 'intron.'
30. Given the general applicability of the teachings of the specification to any potential target gene and any intron, it is my opinion that the '632 application sufficiently described the full range of chimeric DNA constructs recited in the claims. A person of ordinary skill would have recognized that the teachings of the specification could be practiced with more than a sufficient number of known nucleic acid sequences of interest (i.e. target genes) and intron sequences to be representative of the genus as a whole. Therefore, I believe that a person of ordinary skill in the field would have recognized that the applicants were in possession of the full scope of the claimed genus at the time the application was filed.
31. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date:

OCTOBER 30TH 2007

  
Peter Robert Schofield

## BIOGRAPHICAL SKETCH

Professor Peter R Schofield PhD DSc

Executive Director and Chief Executive Officer, Prince of Wales Medical Research Institute  
and Professor of Medicine, University of New South Wales  
Barker Street, Randwick, NSW 2031  
Sydney, Australia.

### EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Sydney, Sydney, Australia	BScAgr HonsI	1982	Genetics
Australian National University, Canberra, Australia	PhD	1985	Genetics
Genentech Inc, South San Francisco, CA, USA	postdoctoral	1985-1987	Molecular Neuroscience
Centre for Molecular Biology, University of Heidelberg, Germany	postdoctoral	1987-1988	Molecular Neuroscience
University of New South Wales, Sydney, Australia	DSc	1998	Medicine

### A. Positions and Honors

#### Positions and Employment

1988-1991	Senior Scientist, Pacific Biotechnology Limited, Rushcutters Bay, Sydney
1991-2006	Garvan Institute of Medical Research, Sydney (1991-93, Senior Research Fellow; 1994-98, Principal Research Fellow; 1999-2005, Senior Principal Research Fellow; 2006 Honorary SPRF)
1993-2005	NHMRC Research Fellowship (1993-1996, Senior Research Fellow; 1997-2000, Principal Research Fellow, Senior Principal Research Fellow 2001-2005)
1999-2004	Director, Neurobiology Research Program, Garvan Institute of Medical Research, Sydney
2000-Present	Professor, School of Medicine, University of New South Wales, Sydney
2004-Present	Executive Director and Chief Executive Officer, Prince of Wales Medical Research Institute, Sydney

#### Other Experience and Professional Memberships

1992 - 2004	Foundation Director, Secretary (1994-2000) & President (2000-04), Genome Conference, Inc.
1995 - 2000	Secretary (1995-2000) & Chairman (2000-04) Garvan Institute, Institutional Biosafety Committee.
1995 - 2005	Member, Pharmaceutical Subcommittee, Australian Drug Evaluation Committee, A statutory committee providing advice to the Federal Minister for Health & Ageing)
1997 - 2002	Director, President-Elect (2000-01), & President (2001-02), The Australian Society of Medical Research
1998 - 2007	Co-Convenor, The Neuroscience Panel, and Member of Scientific Steering Committee NISAD, Neuroscience Institute for Schizophrenia and Allied Disorders
2000 - 2004	Member of Steering Committee and Foundation Director, Research Australia Ltd
2003	Member, Prime Minister's Science, Engineering and Innovation Council (PMSEIC) Working Group "Brain and Mind Disorders: Impact of the Neurosciences"
2004 - 2005	Member, National Neuroscience Consultative Task Force, Invited by the Federal Minister of Health
2004 - 2006	Member, Program Committee, 11 <sup>th</sup> International Congress of Human Genetics
2005	Member, Legislation Review Committee, Prohibition of Human Cloning Act 2002 & Research Involving Human Embryos Act 2002. Appointed by the Federal Minister for Ageing
2007 - present	Member, Research Council, Schizophrenia Research Institute Australia (formerly NISAD)

#### Honors

1982	The University Medal, Faculty of Agriculture, Sydney University
1990	A. W. Campbell Award, Australian Neuroscience Society
1991	Elsie Waltham Thompson Award, National Heart Foundation of Australia
1995	Boehringer-Mannheim Medal, Australian Society for Biochemistry and Molecular Biology Inc.
1997	Gottschalk Medal, Australian Academy of Science

### B. Selected peer-reviewed publications (in chronological order).

(Publications selected from 173 peer-reviewed publications)

- Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rhee LM, Rodriguez H, Ramachandran J, Glencorse TA, Reale V, Seeburg PH and Barnard EA (1987) Sequence and functional expression of the GABA<sub>A</sub> receptor shows a ligand-gated receptor superfamily. *Nature* 328: 221-227
- Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Koehler M, Fujita N, Rodriguez H, Stephenson FA, Darlison MG, Barnard EA & Seeburg PH (1988) Structural and functional basis for GABA<sub>A</sub> receptor heterogeneity. *Nature* 335: 76-79

- Pritchett DB, Sontheimer H, Gorman CM, Kettenmann H, Seeburg PH and Schofield PR (1988) Transient expression shows ligand gating and allosteric potentiation of GABA<sub>A</sub> receptor subunits. *Science* 242: 1306-1308
- Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR and Seeburg PH (1989) A novel GABA<sub>A</sub> receptor subunit important for benzodiazepine pharmacology. *Nature* 338: 582-585
- Vandenberg RJ, French CR, Barry PH, Shire J and Schofield PR (1992) Antagonism of ligand-gated ion channel receptors: Two domains of the glycine receptor  $\alpha$  subunit form the strychnine binding site. *PNAS* 89: 1765-1769
- Vandenberg RJ, Handford CA and Schofield PR (1992) Distinct agonist- and antagonist-binding sites on the glycine receptor. *Neuron* 9: 491-496
- Ryan SG, Buckwaller MS, Lynch JW, Handford CA, Segura L, Shiang R, Wasmuth JJ, Camper SA, Schofield P and O'Connell P (1994) A missense mutation in the gene encoding the  $\alpha 1$  subunit of the inhibitory glycine receptor causes the spasmodic mouse phenotype. *Nature Genetics* 7: 131-135
- Rajendra S, Lynch JW, Pierce KD, French CR, Barry PH & Schofield PR (1995) Mutation of a single amino acid in the human glycine receptor transforms  $\beta$ -alanine and taurine from agonists into competitive antagonists. *Neuron* 14: 169-75
- Lynch JW, Rajendra S, Pierce KD, Handford CA, Barry PH & Schofield PR (1997) Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel. *EMBO J* 16: 110-120
- Baker M, Kwok JBJ, Kucera S, Crook R, Farrer M, Houlden H, Isaacs A, Lincoln S, Onstead L, Hardy J, Wittenberg L, Dodd P, Webb S, Hayward N, Tannenberg A, Andreadis A, Hallupp M, Schofield PR, Dark F & Hutton M (1997) Localisation of fronto-temporal dementia with Parkinsonism in an Australian kindred to chromosome 17q21-22. *Annals Neurology* 42: 794-798
- Adams LJ, Mitchell PB, Fielder SL, Rosso A, Donald JA and Schofield PR (1998) A susceptibility locus for bipolar affective disorder on chromosome 4q35. *American Journal of Human Genetics* 62: 1084-1091
- Hutton M, et al (1998) Coding and 5'-splice-site mutations in tau with inherited dementia FTDP-17. *Nature* 393: 702-5
- Kwok JBJ, Li Q-X, Hallupp M, Whyte S, Beyreuther K, Masters CM and Schofield PR (2000) Novel Leu723Pro APP mutation increases A $\beta$ 42(43) peptide levels and induces apoptosis. *Annals of Neurology* 47: 249-253
- Stanford PM, Halliday GM, Brooks WS, Kwok JBJ, Storey CE, Creasey H, Morris JGL, Fulham M and Schofield PR (2000) Progressive supranuclear palsy pathology caused by a novel silent mutation S305S in exon 10 of the tau gene: Expansion of the disease phenotype caused by tau mutations. *Brain* 123: 880-893
- Smith MJ, Kwok JBJ, McLean CA, Kril JJ, Broe GA, Nicholson GA, Cappai R, Hallupp M, Cotton RGH, Masters CL, Schofield PR and Brooks WS (2001) Variable phenotype of Alzheimer's disease with spastic paraparesis. *Annals of Neurology* 49: 125-129
- Lynch JW, Han N-LR, Haddrell J, Pierce KD and Schofield PR (2001) The surface accessibility of the glycine receptor M2-M3 loop is increased in the channel open state. *Journal of Neuroscience* 21: 2589-2599
- Badenhop RF, Moses MJ, Scimone A, Mitchell PB, Ewen KR, Rosso A, Donald JA, Adams LJ and Schofield PR (2001) A genome screen of a large bipolar affective disorder pedigree supports prior evidence for a susceptibility locus on chromosome 13q. *Molecular Psychiatry* 6: 396-403
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- Kwok JBJ, Kapoor R, Gotoda T, Iizuka Y, Yamada N, Kato N, Isaacs K, Kushwaha V, Church WB, Schofield PR and Kapoor V (2002) A missense mutation in kynurenine aminotransferase-1 in spontaneously hypertensive rats. *Journal of Biological Chemistry* 277:35779-35782
- Stanford PM, Shepherd CE, Halliday GM, Brooks WS, Schofield PW, Brodaty H, Martins RN, Kwok JBJ and Schofield PR (2003) An increase in three repeat tau causes frontotemporal dementia. *Brain* 126: 814-826
- Kwok JBJ, Hallupp M, Halliday GM, Brooks WS, Dolios G, Murayama O, Badenhop RF, Wang R, Gandy SE, Vickers J, Takashima A and Schofield PR (2003) Presenilin-1 mutation Leu271Val results in altered exon 8 splicing and Alzheimer's disease with non-cored plaques and no neuritic dystrophy. *J Biol Chem* 278: 6748-6754
- Brooks WS, Kwok JBJ, Kril JJ, Broe GA, Blumbergs PC, Tannenberg AE, Lamont PJ, Hedges P and Schofield PR (2003) Alzheimer's disease with spastic paraparesis and "cotton wool" plaques: two pedigrees with PS-1 exon 9 mutations. *Brain* 126: 783-791
- Segurado R, et al. (2003) Genome Scan Meta-Analysis of Schizophrenia and Bipolar Disorder Part III: Bipolar Disorder. *American Journal of Human Genetics* 73: 49-62
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Gregory GC, Macdonald V, Schofield PR, Kril JJ and Halliday GM (2006) Differences in regional brain atrophy in genetic forms of Alzheimer's disease. *Neurobiology of Aging* 27: 387-393

Graham BA, Schofield PR, Sah P, Margrie TW & Callister RJ (2006) Distinct physiological mechanisms underlie altered glycinergic synaptic transmission in the murine mutants *spastic spasmodic* and *oscillator*. *J Neuroscience* 26: 4880-90

Pickering-Brown SM, Baker M, Gass J, Boeve BF, Loy CT, Brooks WS, McKenzie IRA, Martins RN, Kwok JBJ, Halliday GM, Kril J, Schofield PR, Mann DMA and Hutton M (2006) Mutations in *progranulin* explain atypical phenotypes with variants in *MAPT*. *Brain* 129: 3124-3126

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Boucher AA, Arnold JC, Duffy L, Schofield PR, Micheau J and Karl T (2007) Heterozygous neuregulin1 mice are more sensitive to the behavioural effects of  $\Delta^9$ -tetrahydrocannabinol. *Psychopharmacology* 192: 325-336

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Karlstrom H, Kwok JBJ, Gregory GC, Hallupp M and Schofield PR (2007) No association of spastic paraparesis genes in PSEN1 Alzheimer's disease with spastic paraparesis. *NeuroReport* 18: 1267-1269

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Graham BA, Brichta AM, Schofield PR and Callister RJ (2007) Altered potassium channel function in the superficial dorsal horn of the *spastic* mouse. *The Journal of Physiology* (in press)

Rademakers R et al. (2007) An international initiative to study phenotypic variability associated with progranulin haploinsufficiency in patients with the common c.1477C>T (p.R493X) mutation. *The Lancet Neurology* (in press)

### C. Research Support.

#### Current Support

NHMRC = Australian National Health and Medical Research Council (NIH equivalent)

ARC = Australian Research Council (NSF equivalent)

NHMRC/ARC "Ageing Well, Ageing Productively" Program Grant No 401162 Sachdev (PI) 1/1/2007-12/31/2011

"Gene-Environment interactions in healthy brain ageing and age-related neurodegeneration"

Role: Co-investigator

Study: A newly established collaborative study to recruit and longitudinally follow older Australian Twins for markers of healthy ageing and identify gene-environment interactions that lead to neurodegeneration.

JO & JR Wicking Trust

Schofield (PI)

1/1/2007-12/31/2009

"An information and support system for families with hereditary dementia – an Australia-wide program"

Role: PI                                      Study: A pilot grant to provide information and support to Australian early onset Alzheimer's disease APP and PSEN1 pedigree members

Australian Rotary Health Research Fund - Grant into Alzheimer's Disease                      Kwok (PI)                      1/1-12/31/2007  
"The role of genetic contributors in the presentation of psychiatric symptoms in Alzheimer's disease"

Role: Co-Investigator                      Study: A pilot grant to identify and recruit research participants with Alzheimer's disease and behavioural symptoms through the use of Medicare health records

ARC Linkage Grant No LP0455104                      Williams (PI)                      11/1/2004-10/31/2007

"Development of integrated biological markers of brain function"

Role: Co-Investigator                      Study: A collaborative grant working with the startup neuroinformatics group Brain Resource Company to examine and correlate genetic markers with phenotypic markers of normal brain function.

NHMRC Project Grant No 376011                      Schofield (PI)                      1/1/2006-12/31/2008

"The biological role of the cadherin gene FAT in bipolar disorder susceptibility"

Role: PI                                      Study: To define the role of the first positionally cloned susceptibility gene for bipolar disorder, FAT, and to identify the biological mechanisms by which the disease associated SNPs may cause disease.

NHMRC Enabling Grant No 401184                      Schofield (PI)                      1/1/2006-12/31/2010

"Genetic Repositories Australia"

Role: PI                                      Study: An 'enabling grant' (research facilities and infrastructure) to establish and operate a national DNA and Cell Repository to facilitate pedigree and population studies

ARC Discovery Project Grant No DP0774248                      Schofield (PI)                      1/1/2007-12/31/2009

"Identification of genetic polymorphisms of synaptically expressed genes that contribute to variation in normal brain function"

Role: PI                                      Study: To identify functional SNPs in synaptically expressed genes and to examine their association with multiple phenotypic measures of normal brain function in a large population cohort.

NHMRC Project Grant No 455310                      Schofield (PI)                      1/1/2007-12/31/2009

"Mechanism of signal transduction and receptor activation in ligand gated ion channel receptors"

Role: PI                                      Study: A structure-function study examining the inhibitory glycine receptor.

#### Past Support

NHMRC Research Fellowship No 157209                      Schofield (PI)                      1/1/2002 – 12/31/2006

"NHMRC Senior Principal Research Fellowship and Support Enhancement Option"

Role: PI                                      Study: This award was a continuation of a career support fellowship at the full professorial level from the Australian NHMRC to support my full time research salary.

NHMRC Project Grant No 276401                      Schofield (PI)                      1/1/2004-12/31/2006

"Identification and characterisation of phenotypic modifier genes in familial Alzheimer's disease"

Role: PI                                      Study: Characterised variable clinical phenotypes in AD and resulted in the identification of a novel kinase that modifies AD genes and a new genetic locus that may encode a disease modifying gene.

NHMRC Project Grant No 230802                      Schofield (PI)                      1/1/2003 – 12/31/2005

"Understanding the molecular basis of bipolar affective disorder"

Role: PI                                      Study: Using linkage, positional cloning and association analysis the project resulted in the successful identification of the cadherin gene FAT as the first positionally-cloned bipolar disorder susceptibility gene.

NHMRC Project Grant No 276403                      Schofield (PI)                      1/1/2004-12/31/2006

"Molecular determinants of inhibitory synaptic function studied using mutant and transgenic mice"

Role: PI                                      Study: Using various and mutant mouse lines, the electrophysiological nature of synaptic inhibition was examined. Provided evidence for a mechanism of functional compensation in synaptic signaling.



## The Nobel Prize in Physiology or Medicine 1993



### Press Release

NOBELFÖRSAMLINGEN KAROLINSKA INSTITUTET  
THE NOBEL ASSEMBLY AT THE KAROLINSKA INSTITUTE

11 October 1993

The Nobel Assembly at the Karolinska Institute has today decided to award the 1993 Nobel Prize in Physiology or Medicine jointly to

**Richard J. Roberts and Phillip A. Sharp**

for their discovery of "split genes".

### Summary

Our knowledge regarding the genetic material, the genes, has increased dramatically during the last forty years due to achievements in the area of molecular biology. During the first decades, studies on simple organisms, in particular bacteria and bacterial viruses, dominated. A gene was conceived as a continuous segment within the very long double-stranded DNA molecules, the chemical substance of heredity. This simple picture of gene structure completely changed when Richard J. Roberts and Phillip A. Sharp in 1977 independently discovered that genes could be discontinuous, that is, a given gene could be present in the genetic material (DNA) as several, well-separated segments. As their experimental model system, both Roberts and Sharp used a common cold-causing virus, called adenovirus, whose genes display important similarities to those in higher organisms. Shortly thereafter it could be shown by several researchers that split genes are frequent in higher organisms, including man.

Roberts' and Sharp's discovery has changed our view on how genes in higher organisms develop during evolution. The discovery also led to the prediction of a new genetic process, namely that of splicing, which is essential for expressing the genetic information. The discovery of split genes has been of fundamental importance for today's basic research in biology, as well as for more medically oriented research concerning the development of cancer and other diseases.

### The genetic material

During the last forty years our knowledge of how the genetic material, the genes, governs the basic activities of life has increased dramatically. This is due to progress made within molecular biology, the area in science which explores biological phenomena and structures at the molecular level. Many of the most important discoveries within this area have been awarded a Nobel Prize. Examples include the discovery of how the nucleic acid DNA, the chemical substance of heredity, is built (1962), how the synthesis of nucleic acids takes place (1959), how the activity of genes is regulated (1965) and what the genetic code looks like (1968). This knowledge evolved primarily through studies of simple organisms such as bacteria and viruses infecting bacteria.

The general concept prevailing during the mid 1970s regarding the hereditary material and its function can be summarized as follows. A gene exists as a continuous stretch (segment) within a long, double-stranded DNA molecule. When the gene is activated, its information is copied into a single-stranded RNA molecule, called messenger RNA, which translates the information into a protein (figure 1A).

This simple picture of the sequence of events radically changed through the discovery made in 1977 by Richard J. Roberts, working at the Cold Spring Harbor Laboratory on Long Island, New York, and Phillip A. Sharp, working at the Massachusetts Institute of Technology in Cambridge, USA. They found that an individual gene can comprise not only one but several DNA segments separated by irrelevant DNA (figure 1B). Such discontinuous genes exist in organisms more complex than those studied earlier.

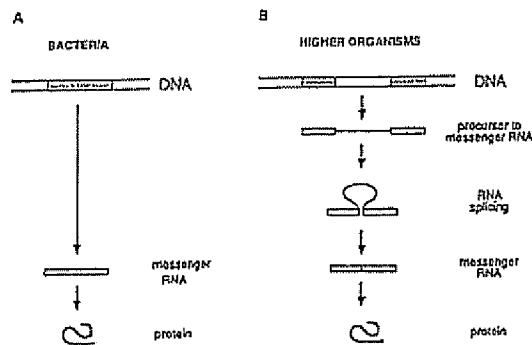


Figure 1: Gene structure and the flow of genetic information in bacteria (A) and higher organisms (B). In bacteria, the genetic information is stored as a continuous segment of DNA, and the messenger RNA can immediately direct the synthesis of the corresponding protein. In higher organisms, the gene is usually split, and the messenger RNA has to be processed by splicing before it can be translated into a protein.

### How the discovery was made

Roberts and Sharp were studying the genetic material in adenovirus, a virus causing common cold. This virus infects the cells of higher organisms, and its genome has many properties resembling those of the host cell. At the same time, adenovirus has a simple structure, making it a very valuable experimental model for studying genes and their function in higher organisms. The genome of adenovirus consists of one single long DNA molecule. Roberts' and Sharp's aim was to determine where in the genome different genes were located.

In biochemical experiments it was shown that one end of an adenovirus messenger RNA did not behave as expected. One of several possible explanations was that the DNA segment corresponding to this end was not located in the immediate vicinity of the rest of the gene. To determine where this segment was located on the long DNA molecule, they used electron microscopy. They surprisingly found that a single RNA molecule corresponded to no less than four well-separated segments in the DNA molecule (figure 2). Roberts and Sharp came to the conclusion that the genetic information in the gene was discontinuously organized in the genome, a conclusion that contradicted the commonly held view regarding the structure of genes. The discovery immediately led to intensive research to find out whether this gene structure is present also in other viruses and in ordinary cells. Very soon after the initial discovery, several researchers could show that a discontinuous (or split) gene structure was common - and in fact the most common gene structure in higher organisms.

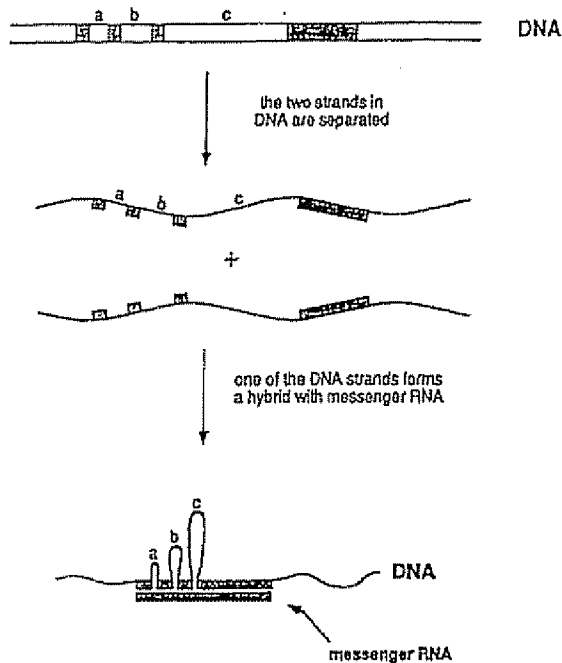


Figure 2: Schematic representation of the experiment that demonstrated that adenovirus DNA contains split genes. The genetic information in the messenger RNA resides in the DNA as four segments, which are separated by three intervening regions (a, b, and c). In the experimentally produced hybrid between one of the DNA strands and the RNA, the intervening sequences in the DNA strand appear as loops, i.e. the corresponding segments lack counterparts in the RNA. The hybrid could be directly visualized in the electron microscope.

## The importance of the discovery

A gene may thus consist of several segments, usually termed *exons* separated by intervening DNA, termed *introns*. This knowledge has radically changed our view on how the genetic material has developed during the course of evolution. It has long been considered likely that evolution takes place as the result of the accumulation of minor alterations in the genetic material (mutations) resulting in a gradual change.

As a consequence of the discovery that genes are often split, it seems likely that higher organisms in addition to undergoing mutations may utilize another mechanism to speed up evolution: rearrangement (or shuffling) of gene segments to new functional units. This can take place in the germ cells through crossing-over during pairing of chromosomes. This hypothesis seems even more attractive following the discovery that individual exons in several cases correspond to building modules in proteins, so-called domains, to which specific functions can be attributed. An exon in the genome would thus correspond to a particular subfunction in the protein and the rearrangement of exons could result in a new combination of subfunctions in a protein. This kind of process could drive evolution considerably by rearranging modules with specific functions.

The discovery that genes can consist of two or more segments immediately led to a prediction with both surprising and important consequences. The first RNA product synthesized containing both exons and introns has to be "edited" such that the introns are cut out and the remaining exons are joined together to form a shortened RNA molecule. It has now been established that this process indeed takes place, and we have already accumulated detailed information on its nature. The process is called *splicing* and in higher organisms it represents an additional step in the transfer of information as compared to what usually occurs in lower organisms (figure 1B). The importance of splicing became particularly apparent, when it was found that it is not always the same segments that are identified as exons and are included in the final RNA molecule. In different tissues or developmental stages, the final RNA molecule may be different due to the utilization of alternative exon combinations. Thus, the same DNA region can in many cases determine the structure of many different proteins. The process is called alternative splicing and represents a fundamentally new principle: the genetic message, which gives rise to a particular product, is not definitely established at the stage when the RNA is first synthesized. Instead, it is the splicing pattern that determines the nature of the final product.

## Medical aspects

Hereditary diseases are common - their estimated number is today no less than about 5000. Some of them are due to errors in the splicing process. The most studied of such diseases is beta-thalassemia, an anemia prevalent primarily in some Mediterranean countries.

The disease is due to a faulty protein, which forms part of hemoglobin in red blood cells. The protein is called beta-globin. If no or badly functioning beta-globin is made, the life-span of the red blood cells is shortened resulting in anemia. In different patients, small defects in the genetic material have been found, resulting in errors in the splicing process and thus in the synthesis of poorly functioning beta-globin. In the upper part of figure 3 the normal splicing of beta-globin RNA is shown (A). If the globin gene is damaged (marked by an arrow) it may, for example, lead to the formation of a larger than normal exon during splicing (B), or to the formation of a completely new exon (C).

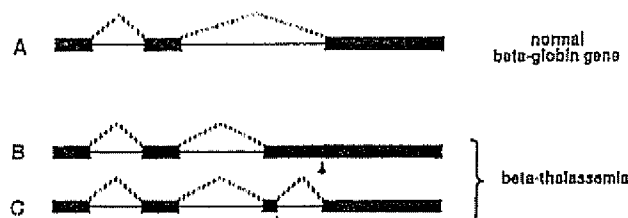


Figure 3: Defective splicing causing beta-thalassemia. A normal beta-globin gene is presented in A, and two mutated genes that result in beta-thalassemia are shown in B and C. Arrows mark the position of point mutations. The interrupted lines denote the segments that are being joined during the splicing process. In the healthy individual, three segments are spliced as shown in A. In one of the thalassemia cases, an unusually long third segment is formed (B), while in the second one, an extra segment is produced (C).

Another example showing the connection between disease and the organisation of the genetic material into exons and introns is chronic myeloid leukemia, a type of cancer of the blood. Characteristic for this disease is the presence in tumor cells of a special, shortened chromosome, called the Philadelphia chromosome, named after the city in which it was discovered. This chromosome has arisen in a white blood cell by fusion of one end of chromosome 22 to one end of chromosome 9. At the break-point, a large portion of a cancer gene has been joined to another gene. Here we are thus dealing with two genes, which are now copied into one single RNA molecule. During the splicing process exons from the two genes are spliced to form an RNA molecule that specifies the synthesis of a new protein, a so-called fusion protein. This new protein gives rise to leukemia.

## References

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E.H. McConkey: Human Genetics. The Molecular Revolution. Jones and Bartlett, Boston, 1993.

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## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
Min-Bo Wang et al.	)	Group Art Unit: 1635
Application No.: 09/287,632	)	Examiner: JANE J ZARA
Filed: April 7, 1999	)	Confirmation No.: 6526
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED PHENOTYPES	)	
	)	
	)	

### DECLARATION BY DR. MICHAEL METZLAFF UNDER 37 C.F.R. § 1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Dr. Michael Metzloff, hereby states as follows:

1. I am a citizen of Germany.
2. I have received a PhD degree from the University of Halle (Germany) in 1983. I am an expert in the field of the plant molecular biology, particularly the field of post-transcriptional gene silencing. I have authored and co-authored several scientific publications in this field including Metzloff et al. 1997 Cell, Vol. 88 pages 845-854, 1997. My curriculum vitae including a list of publications is attached as ANNEX 1.
3. I am currently employed by BAYER BIOSCIENCE N.V., which is a licensee of the US patent application 09/287,632 (referred below as "the Application").
4. I have read the Application.
5. I have also read the USPTO Official Action, dated September 19, 2008 and I understand that the examined claims have been rejected as allegedly being obvious over the combined disclosures of Metzloff et al. (Cell, Vol. 88 pages 845-854, 1997) or Flavell et al. (Proc. Natl. Acad. Sci., Vol 91, pages 3490-3496, 1994) or Stam et al. (Annals of Botany, Vol 79., pages 3-12, 1997) in combination with Brown et al. (US patent 5,859,347) or Lusky et al. (US Patent 6,350,575).

6. I understand that the Brown et al. or Lusky et al. references are not recited in connection with the state of the art in the gene-silencing field and are only relied upon to demonstrate that in certain circumstances introns had been used in expression constructs in the prior art, and I will not further comment on these publications, except to say, that I find no reason in these references that would have led a person of ordinary skill to have included introns in the chimeric constructs that are described and claimed in the application.

7. I also understand that the Examiner is of the opinion that a skilled person would have derived from the Flavell, Metzlaff et al. and Stam et al. publications (or other unnamed contemporaneous publications relating to the field of co-suppression) that expression of target genes in a cell can be inhibited by the introduction of chimeric genes expressing sense and complimentary antisense sequences of the target gene (either from separate constructs or from the same construct) which can form a double stranded RNA molecule.

8. I further understand that the Examiner is of the opinion that a skilled person would then also have been motivated to build further on this alleged knowledge derived from the Flavell, Metzlaff et al. and Stam et al. publications and design constructs wherein complementary sense and antisense strands are expressed as inverted repeats in a single molecule. The Examiner has alleged that *"expression of a single contiguous self annealing construct would provide for more efficient self annealing compared to two separately expressed self annealing molecules, applying scientific logic to the teachings of Flavell, Metzlaff and Stam"*

9. I respectfully disagree with the Examiner for the reasons elaborated below. The publications by Flavell, Metzlaff et al. and Stam et al (or other contemporaneous publications related to the field of co-suppression) did not contemplate that double stranded RNA structures formed between antisense RNA and the sense mRNA could be a triggering agent in gene silencing. A person of ordinary skill in the art understanding the proposed models for gene silencing would not have included a sense and antisense RNA strand in one single molecule to obtain more efficient self annealing. This is because the proposed models and prevailing wisdom considered a antisense strand to be the operative gene-silencing triggering molecule. More efficient self annealing would sequester the antisense strand by base-pairing with the sense strand, which would be contrary to the mechanisms

proposed in the cited papers and generally understood at the time. I was a person of at least ordinary skill at the time and certainly would never have contemplated deliberately introducing complimentary sense and antisense sequence of a target gene which can form a double stranded RNA molecule to increase the efficiency of gene silencing based upon these papers or the general understanding in the art at the time.

10. The application describes methods for reducing the expression of a nucleic acid sequence of interest in eukaryotic cells, such as plant cells, by simultaneously providing the cells with chimeric genes encoding sense and anti sense RNA molecules targeted to the nucleic acid of interest (application page 1, line 5 – Example 2). The sense and antisense RNA molecules may be provided as one RNA molecule (application page 1, line 6- Example 1, page 38-40) that can be transcribed from a recombinant DNA and which is capable of forming an artificial hairpin structure. In its simplest form, the artificial hairpin RNA can be visualized as a double stranded RNA region formed between the complimentary sense and antisense region, and a loop (transcribed from the spacer region) connecting the sense and antisense region. The term “artificial hairpin RNA” refers to the requirement that the hairpin RNA is not naturally occurring in nature, because the sense and antisense regions as defined are not naturally occurring simultaneously in one RNA molecule, or the sense and antisense regions are separated by a spacer region which is heterologous with respect to the target gene, or because the hairpin is not contained within the RNA molecule it is normally associated with (Application page 22, lines 19-28).

11. The gene-silencing methods described in the application were found to be surprisingly more efficient than the gene-silencing methods described in the art, which used either antisense or sense (co-suppression) regions. The higher efficiency of gene silencing can be ascertained by

- a. the greater proportion of the population of transgenic plants in which chimeric genes encoding the artificial hairpin RNA molecules were introduced that exhibited a gene silencing effect; and/or
- b. the greater proportion of the population of transgenic effect that exhibited a very pronounced gene silencing effect.

12. The application also disclosed that inclusion of an intron sequence in the chimeric DNA genes encoding the hairpin RNA molecule enhanced the efficiency of reduction of expression of the target nucleic acid to a surprisingly greater degree (application page 47; application page 23, lines 3-15).

13. This latter invention is recited in the current claims of the application. The claims are thus directed to a chimeric DNA comprising a promoter and a transcription termination and polyadenylation region operably linked to a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest, and wherein the second of the annealing RNA sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of at least part of the nucleotide sequence of the nucleic acid of interest and wherein the DNA region further comprises an intron. Other claims are directed to eukaryotic cell and organisms, such as plants, containing such chimeric genes. The application further contains corresponding method claims, which are however currently withdrawn from consideration.

14. It is my opinion that the application described the invention in sufficient detail to demonstrate that the inventors had a complete conception of the invention and described it sufficiently such that a person of ordinary skill in the art would have understood what the invention was and how it was distinguished over the prior art. It is further my opinion that a person of ordinary skill in the art reading the application would have been able to make and use the invention consistent with the breadth of the claims.

15. As mentioned, such chimeric genes could be used to effect a more efficient silencing of the target gene's expression than the antisense or co-suppression methods described in the prior art. Antisense mediated gene-silencing is the earliest described gene-silencing. In the mid-nineties, it was generally assumed that the antisense RNA formed a double-stranded intermediate with its complementary mRNA resulting in either mRNA degradation by double-stranded RNA specific nucleases, inhibition of RNA processing, transport or translation.

16. In 1990, co-suppression was first described in purple flowered petunia plants genetically modified by the introduction of DNA containing a chalcone synthase coding sequence under the control of the strong CaMV35S. Chalcone synthase is a key enzyme in the flavonoid biosynthesis and therefore in pigment production. Surprising phenotypes were produced in that a very high proportion of the first family of primary transformants had flowers with white sectors, and the flowers of some plants were completely white. It was found that the white sectors of the flowers lacked anthocyanin pigment and contained very low levels of mRNA from both transgene and endogenous chalcone synthase A in petals. In 1995, I co-authored a chapter in a book entitled "Gene silencing in Higher Plants and Related Phenomena in other Eukaryotes" pages 43-58 (Ed. Peter Meyer- Springer Verlag) reviewing mechanisms and hypotheses for co-suppression of chalcone synthase, and other genes in transgenic plants (ANNEX II).

17. As described in that review, four kinds of hypotheses were put forward by the mid-nineties to explain the different gene-silencing phenomena.

18. In the first, inactivation of transcription is postulated due to the physical interaction in the nucleus of the duplicated but non-allelic sequences. Cycles of DNA-DNA or chromatin-chromatin interactions could leave the chromatin or methylation patterns of the participating genes in different states which could consequently interfere with the assembly of essential transcription complexes or the binding of the chromatin to the nuclear matrix.

19. The second hypothesis was based upon elevated competition between the increased number of genes for non-diffusible sequence-specific factors essential for ordered transcription or translation.

20. The third hypothesis focuses on post-transcriptional events. It postulated the degradation of the specific mRNAs due to the synthesis of homologous antisense RNAs in the cell, formation of double-stranded RNAs between the antisense RNA and mRNAs and recognition of the aberrant duplexes as substrates for a RNase. These antisense RNAs could be made from an unknown promoter close to the transgene functioning in the appropriate orientation, possibly by read-through from a neighboring gene (as initially proposed by Grierson, 1991, TIBtech 9, 122-123) or by the action of RNA-dependent RNA polymerase on aberrantly accumulated mRNAs.

21. The fourth hypothesis postulated the inhibition of transcription and/or translation by feedback from a specific gene product that accumulates in aberrantly high concentrations in the transgenic plants, through a self-induced, autoregulated control system.

22. The review ends with a "Concluding perspective" section, wherein we indicated that from surveying the range of examples of gene silencing, it was clear that multiple mechanisms contribute to the observed phenotypes, and that it was desirable not to automatically lump all co-suppression phenomena into a homogeneous group and attempt to find a single mechanism for the observed gene silencing or lack of it. With regard to the co-suppression phenomenon associated with chalcone-synthase in petunia, we further indicated that the data accumulated to that date pointed to association of the phenomena with higher levels of mRNA synthesis and/or antisense RNA.

23. Thus, at that point, we favored the hypothesis that co-suppression was mediated through the involvement of an antisense molecule generated via a unknown mechanism from the sense RNA. The antisense RNA molecule could then form a dsRNA intermediate with the targeted mRNAs which were thus tagged for degradation.

24. In February 1998, Fire et al. published a surprising technique for inhibiting gene function in *C.elegans* by injecting double stranded RNAs corresponding to a target gene in the small worms (Fire et al., 1998; Nature 391, pages 806-811) Although intriguing and efficient, the double stranded RNA hypothesis posed a puzzle of how to reconcile all previously obtained data regarding antisense and sense mediated gene-silencing. As indicated in the editorial comment by Wagner and Stam in the same Nature issue (Wagner and Stam, Nature 391, pages 744-745, ANNEX III), a lot of questions remained unresolved: "*Fire and colleagues have uncovered a complex and intriguing mode of regulation in C. elegans....Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene?*"

25. It is currently understood that dsRNA is a gene-silencing triggering molecule, (which may be generated during viral replication, or by synthesizing a complementary strand on aberrant sense or antisense RNA molecules through the action of a RNA dependent RNA

polymerase). The dsRNA molecule is cleaved by a dsRNA specific RNase (called Dicer or Dicer-like) into smaller dsRNA molecules of 21-24 nt long. The "antisense" strands of these smaller dsRNA molecules are loaded onto a RISC protein complex and act as a guidance molecule to direct the degradation of the corresponding mRNA.

26. I will now specifically address the publications relied on in the Office Action. Starting with Flavell (1994), I disagree with the Examiner's analysis (Office action at page 6) that this publication discloses "*plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation region and further comprising a DNA region encoding a region capable of forming a double stranded RNA stem by base pairing between regions with a sense and antisense nucleotide sequence, which sense nucleotide sequence includes at least 10 consecutive nucleotides having 100% sequence identity with at least 10 consecutive nucleotides having 100% sequence identity with said at least 10 consecutive nucleotides of the sense sequence*" I cannot find either at the indicated passages or anywhere else a disclosure of such structures. Moreover, this analysis by the Examiners seems to be contradictory to the Examiner's conclusion that the "*primary references of Flavell [...] [does] not teach double stranded hairpin constructs in their inverted repeats*" (Office action at page 7, last sentence).

27. Flavell reviews four categories of explanation of the gene-silencing phenomenon that were proposed around 1994 including

- a. Adaptation of an epigenetic state that affects gene expression
- b. Competition between genes for nondiffusible factors essential for ordered transcription or translation
- c. Production of unintended antisense formation and the degradation of mRNA sense-antisense duplexes.
- d. Accumulation of higher levels of a specific RNA and degradation of all this mRNA species by some unknown mechanism (page 3492, first column).



Of these 4 categories, only the third one involves the formation of a double stranded RNA intermediate, however this duplex RNA is proposed to form between the produced antisense RNA and the sense messenger RNA and trigger the degradation of the sense messenger RNA. Note that in this model the double stranded RNA molecule is thus not made between an antisense RNA region and an sense RNA region, different from the target mRNA to be degraded. According to this model, the antisense RNA is the pivotal molecule in gene-silencing and may be introduced intentionally or unintentionally (via transcription under control of promoters outside the transgene or through the action of RNA dependent RNA polymerase on aberrant RNA templates). In view of this model, it would not be logical to enhance the efficiency of gene silencing by simultaneous introduction of a sense and antisense RNA molecule capable of forming a duplex RNA with each other, since this sense RNA molecule would "compete" with the targeted mRNA molecule for duplex formation with the active antisense molecule triggering the gene-silencing phenomenon. The competition would even be more severe if the introduced sense and antisense RNA would be present in one molecule, as such intramolecular duplex formation would be favored over intermolecular duplex formation.

28. Turning to Stam et al., I also disagree with the Examiner's analysis (Office action at page 7) that this publication discloses "*plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation region and further comprising a DNA region encoding a region capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base pairing between regions with a sense and antisense nucleotide sequence, which sense nucleotide sequence includes at least 10 consecutive nucleotides having 100% sequence identity with at least 10 consecutive nucleotides having 100% sequence identity with said at least 10 consecutive nucleotides of the sense sequence*". Again, I cannot find at the indicated passages or anywhere else a disclosure of such structures.

29. Stam et al. review potential models to explain posttranscriptional gene silencing. As indicated in the legend of Figure 1, "the key features considered are: the production of aberrant transcripts, the activity of host encoded RNA-directed RNA polymerase and the production of complementary RNA (cRNA or antisense RNA)". Thus, as elaborated with regard to Flavell (paragraph 11), Stam et al attribute a central role in gene

silencing to the antisense RNA molecule. The mentioned repeat structures are not within a single gene (as contemplated by the Application) but are with regard to entire transgenes which can be in inverted and direct repeat. According to Stam et al. (page 8) such a multicopy locus is prone to deliver the hypothetical aberrant RNA which through the action of RdRP could yield complementary RNAs which can hybridize to the (sense) mRNA and be degraded. Again, as elaborated above for Flavell, Stam et al. disclose that double stranded RNA structures formed between antisense RNA and the sense mRNA can be involved as an intermediate in gene silencing but are not described as a triggering agent.

30. Metzlaff et al. is a publication that I co-authored with Flavell and others. It reports work performed in the labs of Richard Flavell. In particular, we analyzed the presence of shorter poly(A)- and poly(A)+ RNAs in petunia plants where the chalconsynthase expression is reduced through the introduction of a sense chalconsynthase gene under control of a CaMV 35S promoter, resulting in white flowers or flowers with white sectors. As explained above in paragraph 11, our working hypothesis was that sense RNA mediated co-suppression involved generation of an antisense RNA which could hybridize with the messenger RNA leading to degradation of both endogenous and transgene chalcone synthase mRNA. The model presented in Metzlaff et al. also involves complementary RNA, but in the case of chalconsynthase RNA the complimentary RNA is inherent in the sense transcript (see discussion last sentence) as the *chs* mRNA naturally contains complementary sequences (43 bp which are 80% complementary and are located at the 3' end of coding sequence of *chs* gene and in the 3'untranslated region). It will be clear that the last sentence in the abstract concerning the presentation of "*a model involving cycles of RNA-RNA pairing between complementary sequences followed by endonucleolytic RNA cleavages*" refers to the model schematically presented in Figure 7, where a duplex RNA molecule is formed between the aberrant RNA *chs* molecules and the complementary region elsewhere in the *chs* mRNA to initiate endonucleolytic cleavage resulting in further aberrant RNA *chs* derived molecules, perpetuating the cycle of duplex formation with the mRNA and cleaving thereof.

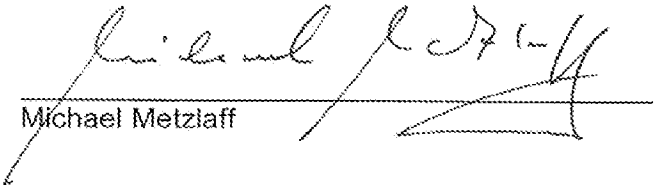
31. In conclusion, Flavell, Stam and Metzlaff all emphasize the importance of the complementary RNA/antisense RNA as the central effector molecule in gene silencing. I

reiterate that accordingly it would not be logical to enhance the efficiency of gene silencing by simultaneous introduction of a sense and antisense RNA molecule capable of forming a duplex RNA with each other, since this sense RNA molecule would compete with the targeted mRNA molecule for duplex formation with the active antisense molecule triggering the gene-silencing phenomenon. The competition would even be more severe if the introduced sense and antisense RNA would be present in one molecule, as such intramolecular duplex formation would be favored over intermolecular duplex formation.

32. It is therefore my opinion that it would not have been obvious to one of ordinary skill in the art to derive from the Flavell, Metzlauff et al. and Stam et al. publications (or other contemporaneous publications in the field of co-suppression in plants) that expression of target genes in a cell can be inhibited by the introduction of chimeric genes expressing sense and complimentary antisense sequences of the target gene (either from separate constructs or from the same construct) which can form a double stranded RNA molecule.

33. I hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date:

18/03/2009  
\_\_\_\_\_  
Michael Metzlauff

# ANNEX I

# ANNEX I

## Curriculum Vitae: Michael Metzlauff

- 2008 - Research Liaison Manager Bayer BioScience  
Management of Research Collaborations between Bayer BioScience and Academia
- 2003-2008 Group Leader and Program Leader at Bayer BioScience N.V. Gent, Belgium  
Administrative tasks: steering and coordination of Bayer's Crop Productivity Research  
Own research: molecular genetics of abiotic stress tolerance, gene silencing/RNAi
- 1999–02 Senior Scientist at Plant Genetic Systems N.V., Aventis CropScience N.V. and Bayer BioScience N.V. carrying out research in virus- and transgene-induced gene silencing
- 1993-98 Senior Scientist at John Innes Centre, Norwich, UK, Department of Genetics working together with Dick Flavell in the field of plant gene silencing
- 1987-92 Lecturer in Genetics at Martin-Luther-University Halle, Germany  
Teaching: Plant Molecular Genetics, Genetic Engineering  
Research: organelle-nucleus interactions in plants, molecular organization of non-coding repetitive sequences, molecular marker-assisted plant breeding
- 1987 Habilitation (Dr.rer.nat.habil.) in Plant Genetics at Martin-Luther-University
- 1983-86 Group Leader at Martin-Luther-University, Department of Genetics  
Research: molecular organization of nuclear and organelle genomes in higher plants of the genera *Pelargonium*, *Antirrhinum*, *Lycopersicon*, *Beta*, *Triticum* and *Secale*
- 1980-83 PhD student at Martin-Luther-University, Department of Genetics  
Topic of thesis: chloroplast DNA variation in the genus *Pelargonium*
- 1975-80 Studies in Biology at Martin-Luther-University Halle, Germany
- 1954 Born in Genthin, Germany on November 26
- Publications: - approx. 50 publications in peer-reviewed scientific journals  
- contributions to several scientific books  
- numerous invited talks at scientific meetings  
- regular reviewing of thesis, manuscripts and research projects at national and international level

## **Publication list Michael Metzlaff**

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# **ANNEX II**

197

# Current Topics in Microbiology and Immunology

*197*

## Editors

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M. Cooper, Birmingham/Alabama · H. Koprowski,  
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# Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes

Edited by P. Meyer

With 17 Figures



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DR. PETER MEYER  
Max-Delbrück-Laboratorium  
in der Max-Planck-Gesellschaft  
Carl-von-Linné-Weg 10  
50829 Köln  
Germany

*Cover Illustration:*

*The front page shows three different examples of gene silencing phenomena in plants: (1) Silencing of a transgenic pigmentation marker in petunia flowers due to DNA methylation (background photo, provided by Iris Heidmann). See p. 15 for further details. (2) Inhibition of tomato fruit ripening by antisense technology (photos in the upper two panels, provided by Don Grierson). Wild-type tomatoes (right) and antisense transformants (left) are shown. See p. 77 for further details. (3) Silencing of anthocyanin pigmentation in maize anthers by paramutation at the *P1* locus (photos in the lower two panels, provided by Garth Patterson). The *P1*-Rh phenotype (right) and the *P1*'-mah phenotype (left) are shown. See p. 121 for further details.*

*The photo on the back page shows examples of flower phenotypes derived from co-suppression of a gene of the pigmentation pathway in petunia (photo provided by Richard Flavell). See p. 43 for further details.*

*Cover design: Künkel + Lopka, Iivesheim*

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# Developmental Regulation of Co-suppression In *Petunia hybrida*

R.B. FLAVELL<sup>1</sup>, M. O'DELL<sup>1</sup>, M. METZLAFF<sup>1</sup>, S. BONHOMME<sup>1,2</sup>, and P.D. CLUSTER<sup>1</sup>

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2 Co-suppression and Plant Development .....	44
3 Hypotheses to Explain Gene Silencing .....	46
4 Mechanisms and Hypotheses for Co-suppression of Chalcone Synthase in Transgenic Petunias .....	49
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## 1 Introduction

The gene silencing phenomenon to be discussed here, initially termed "co-suppression" (NAPOLI et al. 1990; VAN DER KROL et al. 1990; JORGENSEN 1990), was observed in purple-flowered petunia plants genetically modified by the introduction of DNA containing a chalcone synthase coding sequence under the control of the strong CaMV 35S promoter and the 3' end from the nopaline synthase gene of *Agrobacterium*. The selectable marker gene consisting of the coding sequence for neomycin phosphotransferase under the control of nopaline synthase promoter and with the 3' end from the octopine synthase gene was also inserted on the same T-DNA. These genes were introduced into petunia cells via the transferred portion of the Ti plasmid of *Agrobacterium tumefaciens* (i.e. the T-DNA).

Chalcone synthase is a key enzyme in flavonoid biosynthesis and, therefore, in pigment production. These pigments are synthesized intensely in the epidermis of flower petals, but also to lesser extents in many other parts of the plant including the anthers. Pigment production is cell-type specific. Chalcone synthase gene expression is transcriptionally regulated but separate post-transcriptional effects have been described that influence the pigmentation pattern in flowers (MOL et al. 1983). In petunia, chalcone synthases are encoded by a gene family (Koes et al. 1989), and the cDNA used to create the new transgene was

<sup>1</sup> John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK

<sup>2</sup> Present Address: INRA Centre de Versailles, Station de Genetique et Amelioration des Plantes, 78026 Versailles Cedex, France



from the chalcone synthase A (CHS A) allele responsible for most of the chalcone synthase activity in petals (Koes et al. 1989). Surprising phenotypes were produced in that a very high proportion of the first family of primary transformants had flowers with white sectors, and the flowers of some plants were completely white (NAPOLI et al. 1990). This was interpreted to imply that the introduction of a new chalcone synthase gene had caused the loss of most or all chalcone synthase activity from the inserted transgene and the endogenous chalcone synthase genes in the white petal sectors. This interpretation was confirmed by the correlation between the lack of anthocyanin pigment and the very low levels of mRNA from both transgene and endogenous CHS A in petals (NAPOLI et al. 1990; VAN DER KROL et al. 1990). This suppression of both kinds of homologous gene was the reason for using the term co-suppression to describe the phenomenon (NAPOLI et al. 1990). Subsequent analyses of large numbers of transformants and their progeny from the selfing and backcrossing of selected transgenic lines have revealed numerous important features about the co-suppression phenomenon (JORGENSEN 1993b, 1994, and unpublished results).

## 2 Co-suppression and Plant Development

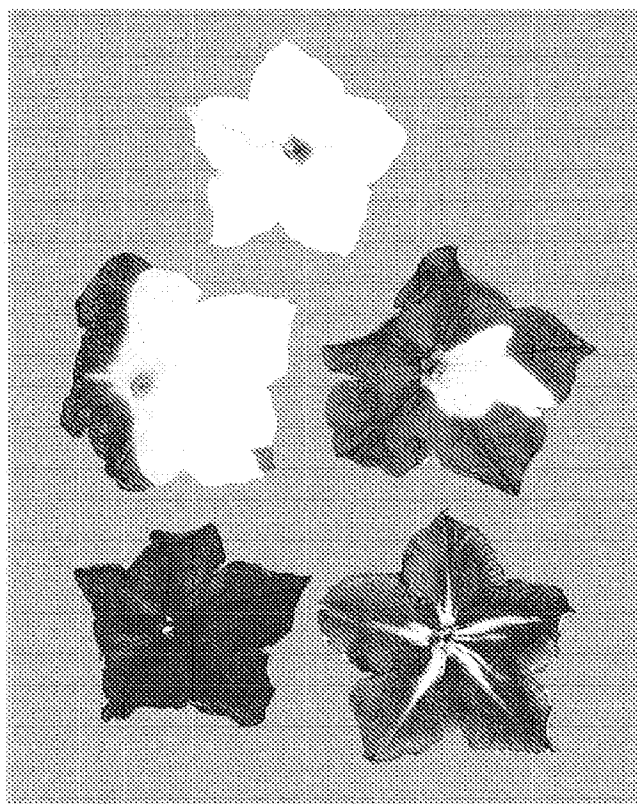
The flower phenotypes showing co-suppression have been classified on the basis of the position and extent of pigmentation in the flowers (JORGENSEN 1993a,b). This classification is meaningful because phenotypes are characteristic for particular transformants even though new variants may arise, as described below. Some of the phenotypes are shown in Fig 1. They range from completely white where pigment production is suppressed in all parts of the flower—tube, corolla and anthers—to other patterns where the white segments are small. In one pattern the pigmentless sector is confined to the tube and the anthers, but frequently extends just outside the tube and to a greater extent on the lower petals. In others pigment loss occurs in small sectors along the veins and/or petal tips. In another pattern, pigment loss is orientated along the edges of the petals. The areas without pigment can be much larger in some phenotypes (NAPOLI et al. 1990). In yet another series of plants, the white sectors are small and dispersed across the flower in complex patterns. All these patterns point to inherent features of flower development that are revealed by the transgene. The cells which lack pigment are not simply clonally related. Instead it appears that cells occupying certain locations in the floral meristem with respect to architectural features of petal shape such as lines of symmetry, respond similarly (but not identically) from petal to petal to the presence of the transgene, and these responses are different from those of other cells in other positions. The pattern boundaries are coincident in the upper and lower epidermis. Thus pattern formation may also require intercellular communication.

The untransformed parent plants show no evidence of such pigmentation patterns, though other varieties do (Red Star and Velvet Picotee; MOI et al. 1983;

possible for most of the chalcone  
 arising phenotypes were pro-  
 duced by primary transformants  
 some plants were completely  
 imply that the introduction of a  
 loss of most or all chalcone  
 of the endogenous chalcone  
 interpretation was confirmed  
 in pigment and the very low  
 of CHS A in petals (NAPOLI  
 of both kinds of homolo-  
 co-suppression to describe the  
 analyses of large numbers of  
 and backcrossing of selected  
 ant features about the co-  
 (and unpublished results).

## Phenotype

have been classified on the basis  
 of patterns (JORGENSEN 1993a,b). This  
 is characteristic for particular  
 patterns described below. Some of  
 the patterns are completely white where  
 the flower—tube, corolla and  
 the anthers, but frequently  
 in the lower petals. In others  
 and/or petal tips. In another  
 of the petals. The areas  
 types (NAPOLI et al. 1990). In  
 all and dispersed across the  
 inherent features of flower  
 cells which lack pigment are  
 cells occupying certain loca-  
 tional features of petal shape  
 not identically) from petal to  
 responses are different from  
 boundaries are coincident in the  
 may also require intercellular  
 presence of such pigmentation  
 (Picottee; Mol et al. 1983;



**Fig. 1.** Flower patterns resulting from insertion of the transgene consisting of the coding sequence of CHS A under the control of the CaMV 35S promoter (see colour version of the figure on the backcover). The untransformed parent has only purple flowers. Phenotypes from top middle round to bottom left display: extensive co-suppression emanating from lower petal junctions, complete co-suppression, co-suppression from lower petal junctions, co-suppression along petal veins

VAN BLOKLAND 1994). From the principles of flower design, one can assume that the architectural basis of the patterns is not caused by the transgene but is an inherent feature of flower development. However, elements of this feature somehow interact with the transgene or its product to produce the observed patterns, and different versions (states) of the transgene interact differently to create the different patterns (JORGENSEN 1993a,b).

The observation that many transgenic plants display a characteristically patterned flower phenotype, based on the patterns of co-suppression, implies that the 'state' of the transgene is somatically inherited. When the meiotic inheritance of transgene effects on flower phenotypes was examined (JORGENSEN 1993b and unpublished), several outcomes were noted. In many cases, the phenotype bred true and is thus germinally stable. In other plants examined, a new range of somatically inherited phenotypes was observed. For example, from

a backcross between a white transformant containing two tandem copies of the new genes and its untransformed parent, many phenotypes were obtained including fully purple, fully white and various patterned types. In these cases the phenotype based on the floral positions of cells showing co-suppression is germinally unstable and the transgene presumably alters its state. Thus it can be concluded that a given transgene can exist in different epiallelic states, and these states can change during meiosis or early embryonic development (JORGENSEN 1993b). Occasionally a lateral branch emerges that displays a different flower phenotype with more or less pigmentation, and the variation is inherited, implying that a change has occurred in the L2 layer of cells in the flowers (JORGENSEN 1994 and personal communication).

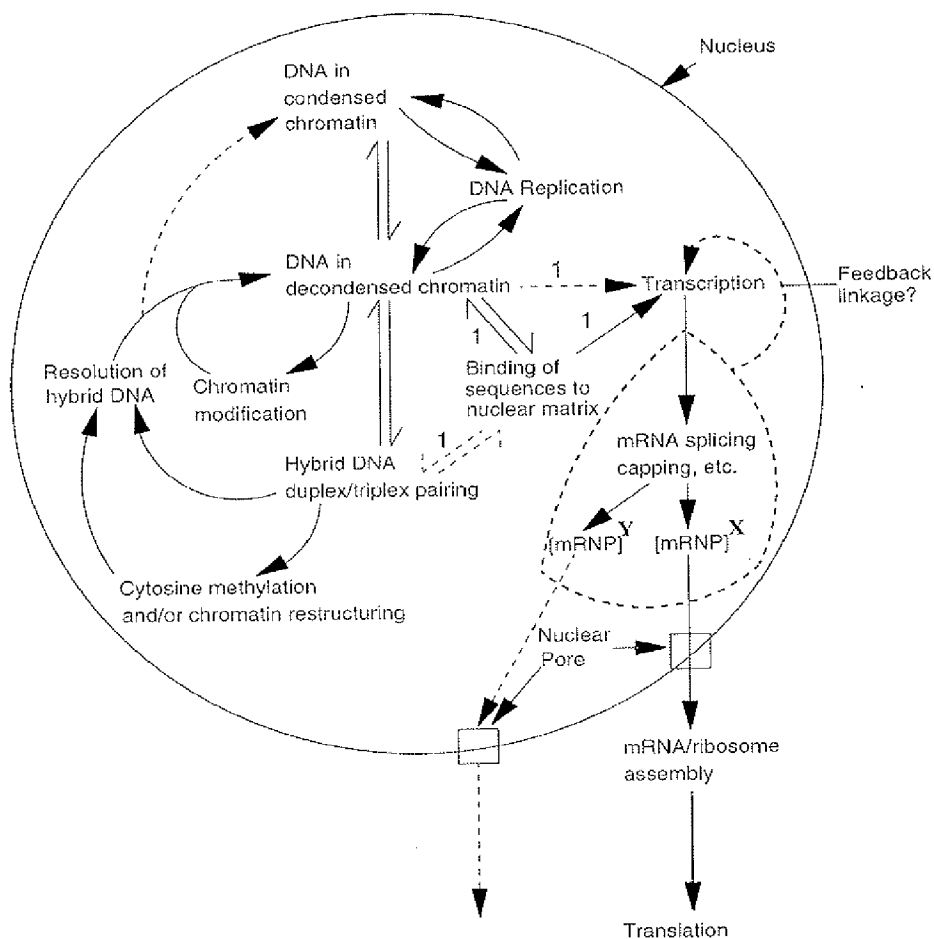
Petunias produce flowering branches from organised groups of cells (meristems) in the axils of leaves or on the flanks of meristems. A genetically different branch results if the group of cells in the meristem flank becomes modified. Occasionally single variant flowers, gradients of phenotypic change as a branch ages, and simultaneous changes in different branches have been noted (JORGENSEN 1994) implying that changes can occur in any floral meristem. Because the inherited L2 layer and the L1 layer in which epidermal pigment is produced are separate developmental lineages of cells, it is reasonable to consider the possibility that the changes in transgene state behind pattern changes occur in many cells of a meristem essentially simultaneously.

The remainder of this chapter deals with the origins of the pigmentless phenotype created by the insertion of the CHS A coding sequence under the control of the CaMV 35S promoter.

### 3 Hypotheses to Explain Gene Silencing

Numerous examples are known, in at least six plant species, where gene inactivations results from the introduction of additional homologous sequences. These have been reviewed elsewhere (JORGENSEN 1990, 1991, 1992; MOL et al. 1991; KOOTER and MOL 1993; MATZKE and MATZKE 1993; MATZKE et al. 1993; ASSAAD et al. 1993; VAUCHERET 1993; GORING et al. 1991; MEYER et al. 1993; GRIERSON et al. 1991; FLAVELL 1994; HART et al. 1992; MEINS 1980; MEINS and KUNZ 1994), and in other chapters in this book (for example see Hamilton et al. and de Lange et al., this volume). They will not therefore be discussed extensively here. However, it should be noted that no single mechanism can explain the variety of examples where loss of gene expression has occurred.

Four kinds of hypotheses have been put forward to explain the diversity of gene silencing phenomena. In the first, inactivation of transcription is postulated due to the physical interaction (ectopic pairing) in the nucleus of the duplicated but non-allelic sequences (loci). Cycles of DNA-DNA or chromatin-chromatin interactions (see Fig. 2) could leave the chromatin structure or methylation patterns of the participating genes in different states which could consequently interfere with



**Fig. 2.** Cellular processes relevant to models for gene silencing. The network of pre-transcriptional events illustrates how the structure of decondensed chromatin, the substrate for transcription, can be modified by various sorts of changes including cytosine methylation and interactions with homologues including transgenes. The changes could modify decondensed chromatin such that it does not bind properly to the nuclear matrix or bind transcription complexes efficiently. After "normal" transcription mRNA is processed, capped and polyadenylated in messenger RNA nuclear protein particles [mRNP]<sup>Y</sup> which are then exported from the nucleus and the mRNA translated on ribosomes. Where gene silencing is post transcriptional, transgene mRNA processing, splicing, capping or polyadenylation could be aberrant, thereby leading to synthesis of aberrant particles [mRNP]<sup>X</sup>. These might not be translated efficiently and may be substrates for RNAscs. They may also be substrates for antisense RNA formation. In any event they do not give rise to protein product. There is the possibility of aberrant mRNA production influencing transcription. Further details are described in the text

the assembly of essential transcription complexes or the binding of the chromatin to the nuclear matrix. These processes are labelled 1 in Fig. 2. There is no direct evidence for such interactions occurring in plants, but precedents come from studies on fungi and *Drosophila*. In *Neurospora* and *Ascomobolus*, DNA homology-searching processes and hybrid DNA formation have been inferred from the inactivation of duplicated sequences via cytosine methylation in premeiotic cells (SELKER 1990; FOSS and SELKER 1991; RHOUNIM et al. 1992; FAUGERON et al. 1990). In yeast, equivalent frequencies of allelic and ectopic meiotic recombination have also been taken to imply the existence of efficient, generalised, DNA sequence homology searching processes (HABER et al. 1991). In *Drosophila* there are many examples where expression of a gene is influenced by "sensing" the presence of another specific gene after some kind of localised somatic chromosome pairing. The pairing could be mediated via DNA, RNA or transcription complexes. The consequentially altered chromatin, sometimes heterochromatic, state created following the interlocus interactions can be clonally inherited when not disturbed by other events (TARTOF and HENIKOFF 1991; HENIKOFF 1992; PIROTTA 1990; WU 1993; PARO 1990).

The second hypothesis is based upon elevated competition between the increased number of genes for non-diffusible sequence-specific factors essential for ordered transcription or translation.

The third hypothesis focuses on post-transcriptional events. It postulates the degradation of the specific mRNAs due to the synthesis of homologous antisense RNAs in the cell, formation of double-stranded RNAs between the antisense RNA and mRNAs and recognition of the aberrant duplexes as substrates for a RNase. Mutual inactivation of homologous mRNAs can often be achieved by the introduction of antisense gene. Double-stranded RNAs may also inhibit translation if they are formed in the cytoplasm (TEMPLE et al. 1993; CORNELISSEN and VANDEWIELE 1989). Evidence for the existence of dsRNA in plants is however very weak (GRIERSON et al. 1991; JORGENSEN 1991; MOL et al. 1991). These antisense RNAs could be made from an unknown promoter close to the transgene functioning in the appropriate orientation, possibly by readthrough from a neighbouring gene or by the action of RNA-dependent RNA polymerase on aberrantly accumulated mRNAs (LINDBO et al. 1993; FLAVELL 1994). This latter enzyme exists in plant cells.

The fourth hypothesis postulates the inhibition of transcription and/or translation by feedback from a specific gene product that accumulates in aberrantly high concentrations in the transgenic plants. This would constitute a self-induced, autoregulated control system (HART et al. 1992; MEINS 1989; MEINS and KUNZ 1994).

#### 4 Mechanisms and Hypotheses for Co-suppression of Chalcone Synthase in Transgenic Petunias

We now consider the petunia chalcone synthase case in the light of these hypotheses. Throughout this discussion, it is relevant to bear in mind that not all transgenic plants containing CHS A transgenes display co-suppression in the petals, and that in some plants only specific segments of petals show co-suppression. Also, it is important to remember that inherited somatic and meiotic changes can occur to influence the extent to which co-suppression is observed.

In the process of making the transgenic plants, it can be expected that different numbers of T-DNAs become stably inserted into different petunia plants and display different structures. Tandem arrays of T-DNAs are common, as are copies inverted with respect to one another. Genetically unlinked T-DNAs also accumulate. Thus plants with different numbers of active genes are likely to be produced, as noted in other studies on transgenic plants (HOBBS et al. 1993; ASSAAD et al. 1993; SCHEID et al. 1991; LINN et al. 1990). It will be important to investigate thoroughly whether the structure of T-DNA inserts influences the extent of co-suppression and the kinds of flower pattern produced. VAN BLOKLAND (1994) has concluded that phenotypic effects of CHS A transgenes are correlated with the presence of inverted repeats of T-DNA.

Where there are multiple copies of the chalcone synthase transgene then the copies might interact (see Fig. 2) to silence transcription of one another and the endogenous CHS A genes. Such silencing has been recorded for several sorts of transgenes (PEACH and VELTEN 1991; ASSAAD et al. 1993; HOBBS et al. 1990, 1993; ELKIND et al. 1990; LINN et al. 1990; MATZKE et al. 1994b; VAUCHERET 1993). Inverted repeats seem to be more frequently associated with transcriptional silencing (HOBBS et al. 1993). How such physical interactions occur is unknown, but they may be the means whereby one or more of the duplicated sequences gain some methylated cytosines. No evidence for silenced CHS A genes becoming routinely methylated has yet been obtained in investigations of several sites within the coding sequences and promoters.

There is evidence, however, that in some transgenic petunias, CHS A transcription is not blocked in petal cells showing loss of pigment. Run-on transcription assays on nuclei from isolated purple and white petal sectors from the same plant show similar levels of CHS A transgene and endogenous CHS A transcription (VAN BLOKLAND 1994). Furthermore, similar levels of unprocessed nuclear endogenous CHS A transcripts have been detected in flowers of some co-suppressed and non co-suppressed variant plants in our laboratory and in that of Mol and co-workers. The levels of RNA transcribed in isolated petal nuclei are not correlated with the extent of chalcone synthase suppression (VAN BLOKLAND 1994; KOOTER and MOL 1993; MOL et al. 1991). These details are reviewed in another chapter in this book (de Lange et al., this volume). We have also found in some plants that white flower sectors retain high levels of CHS A RNA, making it likely that post-transcriptional losses of functional mRNAs are the cause of or a

major contributor to the co-suppression phenotype. Studies of inactivation of some other transgenes in plants have also concluded that the inactivation is post-transcriptional (SMITH et al. 1990a; DE CARVALHO et al. 1992; BATE et al. 1992; MEINS and KUNZ 1994).

In plants where transcription of the CHS A transgenes is not blocked but steady state functional mRNA levels are very low, then a major cause of co-suppression could be accumulation of excess levels of antisense RNAs to the CHS A mRNA, double-stranded RNA formation and degradation of the duplex RNA (third hypothesis above). The presence of antisense RNA to chalcone synthase has been investigated in transgenic petal tissues differing in co-suppression, i.e. purple and white. Of the particular variants studied by us, most were derived from the same transgenic parent and possess two copies of the transgene in inverted orientation. Reverse transcriptase and primers specific for antisense RNA were used to make DNA copies of RNA in RNA extracts isolated from white or purple sectors. Antisense chalcone synthase RNAs were found in both white and purple flower sectors but only in transgenic plants. It is, therefore, concluded that the antisense RNAs are due to the transgene. The finding that antisense chalcone synthase RNAs are in both white and purple sectors suggest that if antisense RNA is essential for the loss of mRNA and gene expression in this genotype, it is clearly insufficient. Similar conclusions have been drawn by Mol and co-workers (de Lange et al. this volume; VAN BLOKLAND 1994) who used other assays to detect antisense RNA.

How is antisense RNA produced from the transgene, what is its structure, and how does it function? These important questions still have to be examined experimentally. It will be necessary to examine many different transgenic plants with different numbers and kinds of transgene structures since it is not clear how antisense RNA could be produced so efficiently in all transformants (JORGENSEN 1991). Where antisense RNA is not transcribed from defined genomic promoters it could be formed by an RNA-dependent RNA polymerase using sense mRNA as template (LINDSO et al. 1993; FLAVELL 1994).

On simple considerations of how antisense RNA interferes with sense mRNA, it would be assumed that the higher the antisense to sense RNA ratio, the more efficient would be the loss of sense gene expression. Some data in plants to support this have been produced (SMITH et al. 1990b; HAMILTON et al. 1990; CANNON et al. 1990; ROBERT et al. 1989; VAN DER MEER et al. 1992). However, there are many reports of discrepancies between the relative levels of antisense RNA transcripts and loss of sense gene expression (reviewed in de Lange, this volume; CANNON et al. 1990; STOCKHAUS et al. 1990; VAN DER KROL et al. 1988). VAN BLOKLAND (1994) found in petunias transgenic for chalcone synthase that antisense transcription could be high in the absence of co-suppression or vice versa. If antisense RNA is the cause of degradation of CHS A mRNAs, but overall steady state or transcription levels of antisense mRNA do not correlate with co-suppression it must be a small fraction of the antisense RNA that is critical, and this fraction must have efficient access to the unprocessed primary RNA transcripts or mRNAs formed after processing, capping and poly-A tail additions. This implies



that the variation in co-suppression in transgenic plants that makes antisense and sense RNAs could involve variation in the accessibility of antisense and sense RNAs to each other in the nucleus or the cytoplasm (see later).

Post-transcriptional loss of CHS A gene expression and pigment production could, alternatively, be due to the accumulation of excess levels of CHS mRNA and the consequential induction of an mRNA-specific process, that is able to catalyse the inactivation and/or degradation of transgene and endogenous CHS A mRNAs (fourth hypothesis above). This hypothesis includes the notion of critical localised threshold levels of mRNA in a cell. Where mRNA levels are below the threshold, purple pigment is produced; in contrast, when the level is exceeded, active mRNA is lost and no pigment is produced. There are several predictions from this model.

1. In plants where all flower epidermal cells lack pigment, the threshold mRNA concentrations are likely to have been exceeded in all cells of the plant. This is as observed in many white flowered plants where levels of transgene mRNA are very low in stems and leaves, as well as flowers (NAPOLI et al. 1990; this laboratory, unpublished).
2. In plants which have purple flowers with white sectors, the levels of localised active mRNA is likely to be higher than that found in plants that make only purple flowers. This is because in such plants small increases in the level of mRNA accumulated would exceed the threshold more readily and thus lead to the pigment loss. Some evidence has been gained to support this hypothesis in that the levels of transgene CHS mRNA accumulating in leaves of transgenic plants with purple and white flowers is greater than in leaves of transgenic plants forming only purple flowers (unpublished results).

If localised mRNA concentrations are the determinant of the post-transcriptional trigger for co-suppression, then the critical parameters affecting co-suppression would be the rate of transgene transcription and/or changes in the efficiency (rate) of mRNA transport through the nucleus, of export through the nuclear envelope, of binding to the ribosome and of translation. Changes in flower pigment production due to different levels of translatable CHS A mRNA could therefore come about through (a) pre-transcriptional events including changes in the levels of transcription factors, restructuring of chromatin, (and/or) changed cytosine methylation to affect the affinity of the transgenes for transcription complexes as noted earlier (see Fig. 2), and/or (b) changes in the rate of mRNA transport, etc. The latter could result from the transgene altering its position in the nucleus with respect to nuclear transport channels and the supply of protein components of the mRNPs essential for correct mRNA processing, transport and export (FLAVELL 1994). Furthermore, aberrantly high levels of mRNA in the nucleus might lead to mRNP particles with a different complement of proteins (WOLFFE 1994) from those formed when the CHS genes are optimally transcribed for mRNA processing and transport and when nuclear mRNA levels are much lower (see Fig. 2). Such modified mRNPs might not make the mRNAs available for translation.



A growing number of proteins are known that bind to mRNA and prevent translation as part of specific regulatory mechanisms. The studies on the ubiquitous Y box proteins and the FRG Y proteins of *Xenopus* oocytes, in particular, are interesting (WOLFFE 1994). These proteins bind to mRNAs and inhibit their translation (BOUVET and WOLFFE 1994). They will bind to a range of double and single stranded DNAs and RNAs including specific Y box sequences in gene promoters (reviewed in WOLFFE 1994). They also stimulate mRNA synthesis, but not necessarily by binding to the promoter motifs. FRG Y2 (a predominantly nuclear Y box protein) has been immunolocalised to nascent transcripts on lampbrush chromosomes (SOMMERVILLE et al. 1993). These observations are consistent with a direct role for FRG Y2 in packaging mRNA in the nucleus and for somehow linking the competence of the mRNA for translation with its transcription (BOUVET and WOLFFE 1994). Recruitment of some other heterogeneous nuclear (hn) RNP proteins on to pre-mRNA is also dependent on transcription (PINAL-ROMA and DREYFUSS 1992), and there is a growing list of eukaryotic proteins having dual roles in the transcription and translation processes (reviewed in BOUVET and WOLFFE 1994).

If protein-based regulatory systems linking transcription, mRNA packaging and mRNA translatability are present in plants, then they might be responsible for post-transcriptional loss of gene expression following "aberrant" mRNA synthesis from the CHS A transgenes. It is also possible that they could lead to shut down of transcription. This whole area of nuclear biology needs to be explored in the context of understanding how aberrant active CHS A transgenes can promote loss of pigment production in flower petals. While it is possible to imagine how mRNA from an aberrantly located transgene might be sequestered into an inactive mRNP structure, how would this affect mRNAs from the endogenous CHS A genes? Perhaps the protein-mediated regulatory systems could also provoke cross-talk between homologous mRNP complexes and sequester all CHS A RNAs into aberrant mRNPs.

If excess CHS mRNAs accumulate due to higher rates of transcription or low rates of mRNP maturation in the nucleus and/or translation, how are they degraded? This could result from the aberrant mRNPs being recognised by RNases and the RNAs consequently degraded (SACHS 1993; SULLIVAN and GREEN 1993). Alternatively, antisense RNA could be produced on the accumulated mRNA templates by RNA-dependent RNA polymerase, and these double-stranded RNA structures are subsequently degraded. Cycles of RNA production of both antisense and sense RNAs could emerge from this process to provide an autocatalytic system for the production of RNAs. Any such antisense RNAs could, of course, explain loss of both transgene and endogenous CHS A mRNAs.

## 5 Concluding Perspective

From surveying the range of examples of gene silencing, it is clear that multiple mechanisms contribute to the observed phenotypes and in some examples

mechanisms resulting in inhibition of transcription are major determinants, while in others post-transcriptional events occur. This diversity of mechanism may also appear between plants genetically altered by insertion of the same or related transgenes, but at different sites, in different arrangements and with different effective promoter strengths. Thus it is desirable not to automatically lump all petunias involving CHS A transgenes into a homogeneous group and attempt to find a single mechanism for the observed gene silencing or lack of it.

The scenario described above for the post-transcriptional control of chalcone synthase silencing may also be combined with, or lead to, variable patterns of transcription silencing in different genotypes. The data accumulated to date point to association of the phenomena with higher levels of mRNA synthesis and/or antisense RNA, and it has been argued that there are probably more than one cellular pool or RNP package for each of these molecules. The fact that sense mRNAs, may exist in different RNP pools and packages implies that we need to look for the different structural forms that may have different stabilities and opportunities to associate with ribosomes and be translated. Similarly we need to investigate whether different antisense RNPs exist.

If only one of the antisense RNP pools is available to interact with only one of the classes of sense mRNP, then the interacting classes are likely to be degraded in co-suppressed tissues, while other classes or pools might not be. Such discoveries might help explain the lack of correlation between antisense RNA to sense mRNA ratios and co-suppression phenotypes in different plants and tissues.

Hypotheses that propose the formation of different mRNP packages from active transgenes in aberrant nuclear positions and the production of pools of antisense RNA in some cases offer the following sorts of explanations for the origins of purple and white flower sectors: In transgenic plants where CHS A transgene transcripts are efficiently processed, packaged and exported then aberrant mRNPs would not accumulate and so co-suppression would not occur. Such plants would have purple flowers. If antisense RNPs were produced in such plants, the antisense RNA might not be accessible to the sense mRNPs and so the flowers would be purple. If, however, transgene mRNA were processed, packaged and exported inefficiently, due to the location or other features of transgene chromatin, then critical levels of nuclear mRNA would be exceeded, packaging could be aberrant and a different mRNP structure for all CHS A mRNAs might result. Messenger RNA in this structure might not be translated, or might be accessible to RNases and antisense RNAs or to RNA-dependent RNA polymerase that makes antisense RNA. Any of these would result in the formation of white flowers.

These ideas are testable and imply that switches in pigment production during transgenic CHS A plant development could result from (a) a change in nuclear position of the transgene; (b) a change in transcription rates (these would constitute inherited changes in state of a transgene); (c) a change in cell physiology influencing nuclear processing, RNA packaging, export and mRNA translation rates; and/or (d) a change in antisense RNA synthesis. Variation in such parameters would not be surprising during meristem development, growth in

different environments or during specific developmental phases. The patterns in flowers are presumably due to similar changes.

The developmental changes influencing the nuclear metabolism of specific clusters of cells in floral meristems and floral tissues are unknown. However, analyses of the CaMV 35S promoter have revealed that it contains multiple elements that respond differently in different petunia floral tissues, leading to differential transcription (BENFEY and CHUA 1989; BENFEY et al. 1989). Thus the enhanced probability that co-suppression occurs in the flower tube and veins in some transgenic genotypes containing CHS A under the control of the CaMV 35S promoter or in other regions in other genotypes could be due to differential interactions between the promoter and enhancer structures of the promoter and the transcription factor concentrations present in different sectors of the meristem and floral tissues.

In conclusion, it is clear that studies into the origins of co-suppression and gene silencing in general will teach us many new features of cell biology and the control of gene expression. Furthermore, because of the wish to create agriculturally novel transgenic plants, understanding how active transgenes can lead to gene silencing is of considerable commercial interest.

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# **ANNEX III**

named after the place of its invention in Eugene, Oregon. The model is a set of three partial differential equations that describe the reaction-diffusion process. Showalter and colleagues added a term to account for the photosensitive generation of bromide ions, and predict wave propagation patterns remarkably similar to those observed in the experiment. Before this experiment, STSR had been studied only theoretically or by numerical or electronic simulation in one-dimensional sets of coupled<sup>6,7</sup> and uncoupled<sup>8</sup> elements, and in two-dimensional arrays of threshold elements<sup>9</sup>. But those 2D simulations, in spite of their simplicity, mimic all the features of the present experiment.

The implications of the present experiment extend far beyond chemical dynamics. Spiral waves, spontaneously generated by noise, have also been simulated with the Oregonator (Fig. 1b). They are strikingly similar to recent observations of noise-initiated and sustained long-range coherent waves of calcium ions in cultured brain tissue<sup>10</sup> (Fig. 1c) indicating a similar under-

lying dynamical process. The possibility that calcium waves transmit or coordinate information over centimetre distances in glial cell networks (that is, in the brain) has already been suggested, but the role of noise remained obscure. Now that noise-sustained spiral waves have been observed in a well characterized chemical system, we can speculate that spatiotemporal noise may be an important feature of the brain's working. □

Frank Moss is at the Center for Neurodynamics, University of Missouri at St Louis, St Louis, Missouri 63121, USA.

e-mail: mossf@umslvma.umsl.edu

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## Functional genomics

# Double-stranded RNA poses puzzle

Richard W. Wagner and Lin Sun

The human genome is predicted to contain between 50,000 and 100,000 genes<sup>1</sup>. To work out what these genes do, an array of techniques is needed to evaluate the protein-protein interactions and biochemical pathways of any gene product. The nematode worm *Caenorhabditis elegans* is an excellent system for such studies because of its well-understood genetics and development, evolutionary conservation to human genes, small genome size and relatively short life cycle. The 100-megabase-pair genome will be completely sequenced this year, and a total of 17,000 genes have been predicted, many with human counterparts. Approaches used to manipulate gene expression in *C. elegans* include transposon-mediated deletion<sup>2</sup>, antisense inhibition<sup>3</sup> and direct isolation of deletions after mutagenesis<sup>4,5</sup>. Although these methods have proved useful, limitations still exist.

On page 806 of this issue, Fire and colleagues<sup>6</sup> describe a remarkable and surprising technique for inhibiting gene function in *C. elegans*. They turned off a specific gene in progeny worms by microinjecting double-stranded RNA (dsRNA) complementary to the coding region of the gene into the gonads of adult animals. Using a well-characterized gene, *unc-22*, which encodes a non-essential myofibril protein, they showed that injection of dsRNA produced a phenotype

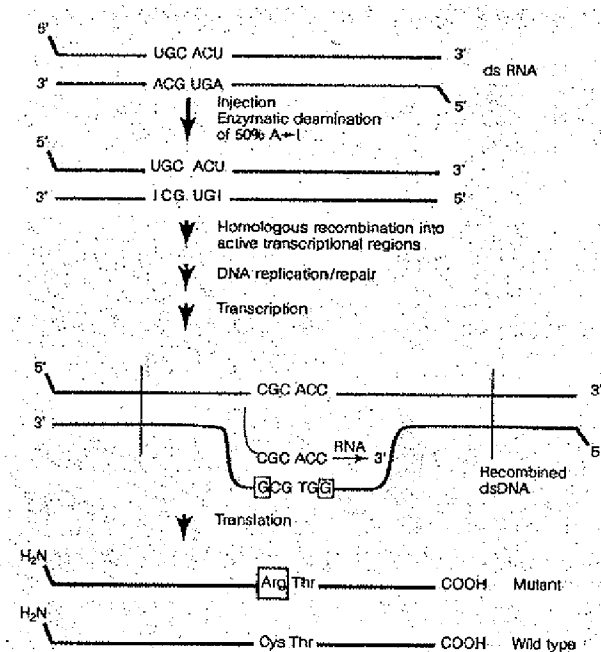
characteristic of *unc-22* inhibition — twitching.

In a series of well-controlled studies, the authors also found that injection of dsRNA targeted to a reporter gene for green fluorescent protein resulted in a dramatic — and

specific — decrease in protein production. Furthermore, when they injected dsRNA targeted to another gene, *mex-3*, the result was a loss of *mex-3* RNA in early-stage embryos. In other words, at the levels of phenotype, RNA and protein, the interference with gene expression was specific and reproducible.

Perhaps most astounding is the phenomenon that the dsRNA causes gene inhibition. Previously<sup>3</sup>, Fire and co-workers had been puzzled by the fact that antisense RNA alone — which is often used to inactivate sense messenger RNA — was only marginally effective. Furthermore, results using the antisense RNA were mimicked by injection of sense RNA, a control in their studies. They later found out that these data could be largely explained by an artefact of the transcription process that was used to generate the antisense and sense RNAs; namely, dsRNA fragments.

Additional experiments by Fire *et al.*, designed to shed light on the possible mechanism of the dsRNA-mediated inhibition, painted an even more mystifying picture. For example, even when only a few copies of the dsRNAs are present in each cell, they are active against highly abundant RNAs. This indicates that the interference occurs either by a catalytic mechanism or at the chromosomal level — and not by a conventional antisense mechanism. The authors also found that only dsRNAs that are complementary to coding regions of the gene are active, and not, for example, those targeted to introns or promoter regions. This argues against a generalized mechanism involving chromosomal inactivation, such as chromosomal deletion. Moreover, dsRNA interference seems to cross cellular boundaries with



**Figure 1** Possible mechanism for inhibition of gene expression in *C. elegans* by double-stranded RNA. Fire *et al.*<sup>6</sup> have convincingly shown that, at the phenotype, RNA and protein levels, dsRNA-mediated interference with gene expression is specific and reproducible. Perhaps, on injection into worms, dsRNA is modified by dsRNA adenosine deaminase. Transfer of this information back into the chromosome may occur by a recombination event. After replication and mismatch repair, transcription and translation result in mutant proteins that have impaired function.

ease. Gene inhibition was observed in progeny when dsRNA was injected into the body cavity of the adult (gonadal injections had been thought to be necessary), and in somatic tissues of young adults after injection into their body cavity.

What kind of mechanism have Fire and colleagues uncovered? This is not the first puzzle posed by dsRNA. Almost ten years ago, Bass and Weintraub<sup>7</sup> and Wagner *et al.*<sup>8</sup> discovered an enzyme that binds dsRNA and deaminates adenosines in the duplex to inosines. After a feverish hunt for the cellular function of the dsRNA adenosine deaminase, it was found to be involved in the post-transcriptional editing of messages. Inosines are read by the cellular machinery as guanosine, so the enzyme could alter the genetic make-up of mRNA (reviewed in refs 9, 10).

Could this dsRNA adenosine deaminase be involved in a complicated pathway that results in gene inhibition in *C. elegans*? Quite possibly. The enzymatic activity has been found in *C. elegans*, and would probably treat the injected dsRNA as a substrate. A specialized homologous recombination system would be needed, which would use the modified dsRNA to transfer the genetic alterations into the chromosome (Fig. 1).

This model fits some of the data: modification of adenosines to inosines alters the genetic make-up of the injected dsRNA; transfer of this information into the genome by recombination would affect coding (but not intronic) regions; and mutations introduced by the inosine substitutions would affect the ability to detect mRNA and, at least partially, the function of the protein. These mutations could account for the surprising result that only a few copies of dsRNA are required per cell, because they would have an effect at the level of the chromosome. Of course, such a model is a stretch of the imagination and is not supported by all of the data. For example, attempts to use homologous recombination with dsDNA in *C. elegans* have largely failed<sup>9</sup>.

Fire and colleagues<sup>6</sup> have uncovered a complex and intriguing mode of regulation in *C. elegans*. Does dsRNA perform a biological function in *C. elegans* (and is this function titrated out by the microinjected dsRNA)? Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene? A similar mode of action would not be suspected to occur in mammals, because injection of dsRNA is often used as a control for antisense experiments, albeit at the individual cell (and not organism) level. Nevertheless, perhaps specific 'knockouts' can be generated this way, for organisms in which genetic material cannot be delivered by microinjection. Whatever the mechanism might be, dsRNA-

mediated inhibition of gene expression will provide a useful alternative for working out gene function in *C. elegans* and, maybe, in other animals and plants.

Richard W. Wagner and Lin Sun are at Phyllos Inc., 300 Putnam Avenue, Cambridge, Massachusetts 02139, USA.

e-mail: rwagner@phyllos.com

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## Liquid crystals

# New designs in cholesteric colour

Peter Palffy-Muhoray

Since their discovery in 1888, cholesteric liquid crystals have been subject to considerable attention, resulting in applications in ink and paint technologies, flat-panel displays and thermal imaging. Writing in *Advanced Materials*<sup>1</sup>, Tamaoki and co-workers describe a new technique for rewritable full-colour image recording on thin cholesteric films. The low-molecular-weight compound they have developed for this purpose is a cholesteric glass, which is stable at room temperature and which could have applications in optics as well as infor-

mation display and storage.

The optical properties of cholesterics have made them useful in display<sup>2,3</sup> and laser technologies<sup>4</sup> as well as in the visual arts<sup>5</sup>. In reflected light, cholesterics show intense iridescent colours with a metallic sheen, as seen on scarab beetles. In these materials, rod-like molecules are orientated, on the average, parallel to one another in a given plane, so that the direction of orientation varies linearly with position in the direction normal to the plane. This results in a spatially periodic twisted helical structure as shown in

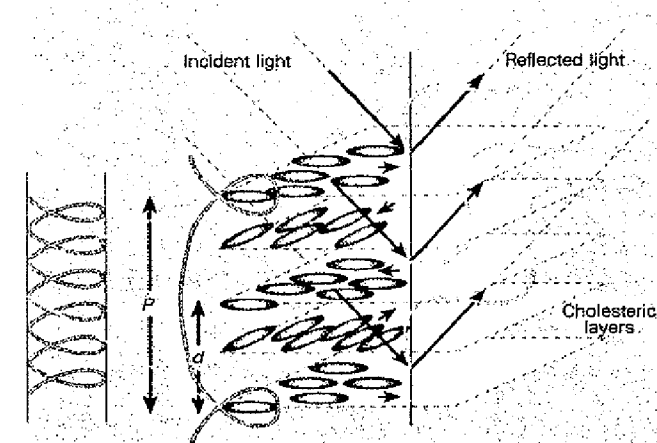


Figure 1 Sketch of cholesteric structure, showing the dependence of molecular orientation on position. The tip of a vector indicating local molecular orientation traces out a helix. Reflected light waves satisfying the Bragg condition emerge in-phase and add constructively. In the work discussed here, Tamaoki *et al.*<sup>1</sup> have developed a cholesteric glass that is rewritable and stable at room temperature (see Fig. 3).



Figure 2 Transmission electron micrograph of freeze-fractured helical cholesteric. The pitch is 240 nm. (From ref. 12.)

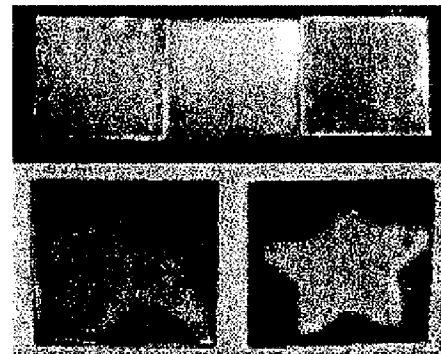


Figure 3 Photographs of thermally addressed and quenched cholesteric solid films. (From ref. 1.)



# Exhibit 23

Attorney's Docket No. 1021565-000060

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
Ming-Bo Wang et al.	)	Group Art Unit: 1635
Application No.: 09/287,632	)	Examiner: JANE J ZARA
Filed: April 7, 1999	)	Confirmation No.: 6526
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED PHENOTYPES	)	
	)	
	)	

## DECLARATION BY INVENTOR UNDER 37 C.F.R. § 1.131

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

We, Peter Michael Waterhouse, Ming-Bo Wang, Michael Wayne Graham and Neil A. Smith, citizens of Australia, hereby state as follows:

1. We are the named inventors of the subject matter of the above captioned application.

2. We have read and understood the Office Action dated November 1, 2007. We understand that U.S. Patent No. 6,506,559 has been cited in the Office Action in rejecting the claims of the present application.

3. We submit Exhibits 1-5 as evidence that embodiments of the presently claimed invention were made, i.e. actually reduced to practice, prior to the December 23, 1997 filing date of U.S. Provisional Application No. 60/068,562 of which benefit is claimed by U.S. Patent No. 6,506,559.

4. Exhibit 1 is a copy of 7 pages from a laboratory notebook prepared by Neil Smith that recorded experiments performed by Neil Smith and/or under his supervision during the construction of a chimeric DNA comprising two copies of a 0.75 kb fragment of the cDNA copy

of the Potato Virus Y RNA genome (PVY) in inverted orientation under control of a CaMV 35S promoter and further comprising intron 2 of the *Pdk* gene from *Flaveria trinervia* in the transcribed region of the chimeric DNA.

5. Notebook page 151 (Exhibit 1, page 1) describes the relevant part of expression vector N2, which was used as starting material to produce the chimeric DNA as described in paragraph 4. That N2 is an expression vector can be seen from the notation ART7, which indicates the backbone of the expression vector pART7 which was commonly used within the CSIRO Plant Industry laboratories at that time. The figure indicates the restriction enzyme sites and denotes the different functional parts of the N2 vector as blocks. The bent arrows indicate the promoter orientation, while the straight arrows indicate the orientation of the PVY inserts. The sizes of the functional parts are indicated between brackets below the blocks and are noted as kilobasepairs. Abbreviations used are: 35S: Cauliflower Mosaic Virus 35S promoter; PVY: approximately 0.75kb fragment of the sequence encoding the protease of potato virus Y; S4: 0.6 kb fragment of the Subterranean Clover Stunt Virus segment S4; Ocs3': 3' termination and polyadenylation signal from the octopine synthase gene from *Agrobacterium tumefaciens*.

6. The notebook page 151 further indicates a first cloning strategy to produce a PVY hairpin RNA having complementary sense and antisense strands targeting the PVY genome part, whereby the 0.6kb HindIII fragment comprising the Subterranean Clover Stunt Virus segment S4 fragment was deleted. In the product, both copies of the PVY fragment are operably linked under the control of one CaMV35S promoter. Upon transcription from the CaMV35S promoter which is active in plant cells, a double stranded RNA molecule as schematically represented ("mRNA double stranded") would be produced. The RNA molecule as represented is an artificial hairpin RNA structure comprising two annealing RNA sequences labeled "sense strand" and " $\alpha$  sense strand", wherein one of the annealing RNA sequences comprises a sense sequence of 750 nucleotides identical to 750 consecutive nucleotides of a PVY target gene of interest in a eukaryotic cell, in this case a plant cell, and the second of the annealing RNA sequences comprises an antisense sequence identical to 750 consecutive nucleotides of the complement of the PVY target gene of interest.

7. Notebook pages 186-188 (Exhibit 1, pages 2-4) describe the preparation of intron 2 of the *pdk* gene of *Flaveria trinervia* and cloning of a PCR amplified fragment having intron 2 (767bp) flanked by restriction enzymes into a small cloning vector (PBC) for amplification purposes.

8. On Notebook page 186 plasmid *pdk1* is described comprising a 10.5 kb *EcoRI* fragment of the *pdk* genomic DNA in pBLUESCRIPT vector. The figure is a schematic representation of the *pdkA* genomic DNA, the cloned *EcoRI* fragment and the position of intron 2. At the bottom is a photo of diagnostic restriction enzyme digests to confirm the structure of *pdk1*.

9. Notebook page 187 represents a nucleotide sequence alignment of the *pdkA* and *pdkB* genes with an indication of the position and nucleotide sequence of intron 2 of *pdkA*. Further indicated are the nucleotide sequences and position of the oligonucleotides used as PCR primers to specifically amplify the intron 2 fragment, flanked by *HindIII* restriction enzyme recognition sites.

10. Notebook page 188 details the PCR reaction protocol to amplify the intron 2 fragment from the plasmid *pdk1*, a gel analysis of the amplified fragment, a ligation protocol for introduction of the PCR- amplified, *HindIII* restricted product into a small vector named PBC and determination of the relative orientation of insertion of the *HindIII* fragment comprising the intron 2 by restriction analysis. At the bottom of the page, the two alternative orientations of insertion into PBC are schematically represented, together with an indication of the expected size of the fragment which was generated after *BglII/PstI* restriction digestion for both orientations and indication of the orientation of inserts in the different clones.

11. The first picture of the gel on Notebook page 189 concerns additional verification for the clones of the intron 2 *HindIII* fragment in PBC in both orientations. Immediately below the

picture is a schematic representation of the cloning strategy to introduce the intron 2 between the two fragments of PVY in inverted orientation under control of a CaMV35S promoter, to generate a double stranded RNA-encoding chimeric gene. To this end, the 0.6 kb HindIII S4 fragment from T-DNA vector pNS2 was replaced by the 0.77 kb HindIII fragment comprising the intron 2 described under paragraph 10. pNS2 comprised the gene components of expression vector N2 described on notebook page 151 (discussed in paragraph 5 above) cloned into T-DNA vector pART27 (see Exhibit 1, page 6 where it is indicated that the backbone vector is pART27, a T-DNA vector commonly used at CSIRO for introduction of chimeric DNAs into plant cells by Agrobacterium-mediated transformation). Notebook page 189 further describes the composition of the ligation mix and the restriction enzyme analysis to identify clones in which the S4 fragment had been replaced by the intron 2 fragment. A small table at the bottom of notebook page 189 indicates the predicted sizes of fragments generated by restriction enzyme digests for each of the two possible orientations of the inserted intron fragment (indicated as "sense intron" and "α-sense intron", the latter is shorthand for intron in antisense orientation).

12. Notebook page 192 (Exhibit 1, page 6) displays the restriction enzyme analysis with Apal enzyme to verify which of the candidate clones contained a single insert of the intron fragment. As indicated next to the gel, the clones with a single insert were expected to produce a restriction fragment of 770 bp, and as stated there "appear to be many [candidate clones] with single Intron 2 inserts". The potentially good chimeric DNA constructs were further verified through XbaI restriction enzyme analysis which as indicated were expected produce a characteristic 2.3 kb fragment. At least candidates "2 and 6 (from pool 7) appear to have [the] intron insert".

13. The bottom part of the Notebook page 192 then represents the restriction enzyme analysis with the restriction enzyme combinations indicated at the bottom of page 189 to determine the orientation of the intron 2 in the chimeric DNA constructs for 4 different clones. The notification under the gel pictures indicate that clone "2" had the intron in antisense orientation, while clones "6", "original single clone 1" and clone "6 from pool 7" had the intron fragment in the sense orientation. Intron 2 is a heterologous intron with respect to the PVY inserts in the chimeric DNA.

14. Notebook page 193 (Exhibit 1, page 7) represents a quality control of a large scale DNA preparation of the T-DNA vector clones 2 and 6 from Notebook page 192. At the top of page 193 it is indicated that the chimeric DNA construct with the intron fragment in antisense orientation is named pNS9, while the chimeric DNA construct with the intron fragment in sense orientation is named pNS10. The bullet point "set up 2 and 6 for tri-parental => LBA4404" refers to the introduction of T-DNA vectors from the *E. coli* host into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid by a process referred to as triparental mating commonly used in the art. The resulting agrobacterial transconjugant strains were cocultivated with tobacco W38 cells in order to introduce the chimeric DNA into plant cells.

15. Exhibit 1 thus describes the successful construction of a chimeric DNA construct molecule comprising, in order:

- a) a promoter, operative in a eukaryotic (plant) cell (*CaMV35S*);
- b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the nucleotide sequence of the nucleic acid of interest (*0.75 kb PVY region in sense orientation- the nucleic acid of interest is thus comprised in the genome of an infecting RNA virus in this embodiment*)); and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*);wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequences; and

wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and

- c) a DNA region involved in transcription termination and polyadenylation (3' *ocs region*).

16. The experiments recorded in Exhibit 1 were completed prior to December 23, 1997.

17. The experiments recorded in Exhibit 1 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

18. Exhibit 2 is a copy of 5 pages from a laboratory notebook (Notebook No 5) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision during the construction of a chimeric DNA encoding an RNA comprising sense and antisense nucleotide sequences targeted to a  $\beta$ -glucuronidase gene (GUS) in which the sense and antisense nucleotide sequences could basepair over about 558 bases, as described in the above mentioned US patent application in Example 1, Figure 1A (6) and Sequence Listing entry SEQ ID NO 1. Further recorded in Exhibit 2 are the introduction of the chimeric gene into a T-DNA vector and introduction of the resulting T-DNA vector into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid. The introduction of the chimeric DNA into plant cells, and data showing reduction of target gene expression in the plant cells are recorded in Exhibits 4 and 5.

19. Exhibit 2, page 1, entitled "To make invert-repeat GUS construct for rice transformation" recorded the starting materials and ligation mixes set up to introduce a second copy of part of the GUS coding region into a vector pWUJGdT comprising a GUS coding region with a deletion of the internal 0.3kb EcoRV fragment.

20. Two cloning strategies were followed i.e.

- a. to introduce a HincII fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 3) into EcoRI restricted vector pWUJGdT (indicated on top of the page as DNA 1 –

treated to render the sticky ends of the restriction fragment blunt and further treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page. This ligation mix is recorded as ligation 2.

- b. To introduce a *Sma*-*EcoRV* fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 4) into *Sma* restricted vector pWUJGdT (indicated on top of the page as DNA 2 – treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page). This ligation mix is recorded as ligation 4.

21. Exhibit 2, page 2 recorded the identification of candidate clones resulting from the cloning strategies, as well as identification of the orientation of the insert into the vector. At the bottom of the page, these two possible insert orientations into the chimeric DNA are schematically represented, resulting in two different vectors namely:

- a. candidate clone 2 (2) renamed pMBW234 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the same orientation as the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml'*).
- b. candidate clone 2 (4) renamed pMBW233 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the inverse orientation relative to the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml'*). Transcription of this chimeric gene yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, namely a sense RNA sequence identical to about 558 consecutive nucleotides of the GUS

gene and an antisense RNA sequence identical to about 558 consecutive nucleotides of the complement of the GUS gene.

22. Exhibit 2, page 3 records the experiments performed to clone the chimeric genes described under paragraph 21, as NotI restriction fragments, into a T-DNA vector designated binary vector pWBVec4A. As indicated in the top right insert, "ligation 2" uses "DNA 1", i.e NotI fragment from pMBW234 ; "ligation 3" uses "DNA 2" i.e NotI fragment from pMBW233.

23. Exhibit 2, page 4 contains the continued recorded data from the experiments described under paragraph 22, including the restriction enzyme analysis, of candidate clones from ligations 2 and 3 mentioned on the previous page. The plasmid denoted pMBW237 refers to the chimeric gene in colony 2(8) [clone 8 of ligation mix 2, thus corresponding to a chimeric gene as in pMBW234], and the plasmid denoted pMBW239 refers to the chimeric gene in colony 3(8) [clone 8 of ligation mix 3, thus corresponding to a chimeric gene as in pMBW233].

24. At the bottom of Exhibit 2, page 4 it is indicated that T-DNA vectors 2(8) and 3(8) were used for triparental mating, referring to the introduction of the T-DNA vectors from the *E. coli* host into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid, and Exhibit 2, page 5 records the verification of the *Agrobacterium* transconjugants as containing the respective T-DNA vectors.

25. The experiments recorded in Exhibit 2 were completed prior to December 23, 1997.

26. The experiments recorded in Exhibit 2 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

27. Exhibit 3 is a copy of a publication by Alan H. Christensen and Peter H. Quail, entitled "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants", which was published in *Transgenic Research* Volume 5, 213-218 (May 1996). This publication describes the ubiquitin promoter region fragment used in the construction of the chimeric genes described in Exhibit 2, as explained under paragraph 21. As indicated in Figure 1, the Ubi promoter region included an intron (Ubi-1 intron) indicated in the Figure by an angled line) in the transcribed region. This publication is mentioned in the above mentioned patent application as a reference for the Ubiquitin promoter used in the chimeric constructs described in Example 1 of that patent



application. At the time of the construction of the chimeric genes, described under paragraphs 20 and 21, we were aware of the presence of an intron in the transcribed DNA region under the control of the Ubi promoter region.

28. Exhibit 4 is a copy of 6 pages from a laboratory notebook (Notebook 96) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision to transform transgenic rice calli containing and expressing a recombinant GUS gene as a target gene of interest in a eukaryotic (plant) cell, with the T-DNA vectors described in Exhibit 2, and to analyse phenotypic expression of GUS in the supertransformed rice calli comprising the target gene and the chimeric DNAs. Data from these analyses correspond to the data recorded in the patent application as "Example 1".

29. Exhibit 4, page 1 indicates that calli were initiated from transgenic rice seeds 4R-V10-28 and 4R-V10-67, both expressing a recombinant GUS gene of interest integrated in the genome of the calli cells. This target gene is a transgene in the cells. The bottom half indicates that these calli were "supertransformed" using different Agrobacteria comprising the different T-DNA vectors including pMBW239 (experiment indicated as "7"). Note also the control experiments where an "empty" control T-DNA vector (pMBW223) was used (experiment 1) and where a conventional co-suppression construct (comprising a transcribed sense nucleotide sequence of the GUSd gene (pMBW225) was used for comparison of the level of phenotypic expression of the target gene (experiment 2).

30. Exhibit 4, page 2 recorded the data for Bialaphos resistant calli obtained with the different Agrobacteria described on the previous page (paragraph 24 above), indicating that such calli had been transformed by the T-DNA's including the chimeric DNA silencing constructs (or control constructs). The transgenic calli obtained by transformation of the initial V10-28 transgenic rice cells with pMBW239 are indicated as "7a" whereas the transgenic calli obtained by transformation of the initial V10-67 transgenic rice cells with pMBW239 are indicated as "7b".

31. Exhibit 4, page 2 at the bottom and continuing on page 3, recorded the data of an initial GUS staining experiment on parts of the transgenic calli that were obtained (V10-67 derived). In control experiment "1b" where the GUS transgenic calli were supertransformed by an "empty" T-DNA vector, all supertransformed calli strongly stained blue indicating strong GUS activity. On page 3 of Exhibit 4, the results of a similar GUS staining on calli obtained by supertransformation with pMBW239 indicating that the observed GUS staining was either weak

("W") , negative ("-") or resulted in an isolated blue spot. These data indicated that the chimeric DNA from pMBW239 reduced the phenotypic expression of the GUS target gene in the rice cells.

32. Exhibit 4, pages 4 and 5 represent the recordation of these data for the individual calli. Exhibit 4, page 6 summarizes the data of an initial GUS staining experiment on parts of the obtained transgenic calli (V10-28 derived). While in series 1a, all calli transformed by the control T-DNA vector pMBW223 strongly stained blue indicating strong GUS activity, the transgenic calli obtained by transformation with pMBW239 (series 7a) all scored as "very weak" or "basically GUS negative" with the exception of only two calli. On the bottom of page 6, reference is made to (quantitative) GUS assays, the data of which are recorded in Notebook 5, starting on page 55, discussed hereinafter as Exhibit 5.

33. The experiments recorded in Exhibit 4 were completed prior to December 23, 1997.

34. The experiments recorded in Exhibit 4 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

35. Exhibit 5 is a copy of 17 pages from a laboratory notebook (Notebook 5) prepared by Ming Bo Wang that recorded quantitative GUS analysis experiments performed by Ming-Bo Wang and/or under his supervision, on the supertransformed rice calli described in Exhibit 4. Particular attention is drawn to the "raw data" concerning GUS analysis by kinetic value measurements on Exhibit 5, page 14 which are represented in Example 1, Table 2 of the above mentioned patent application. See e.g. data for Plate No 3, wells 1A to 2H which have in handwriting indications "7a1" to "7a14" referring to transgenic lines, which are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-28. Similarly, data for Plate No 3, wells 3A to 4D which have in handwriting indications "7b2" to "7b16" referring to transgenic lines are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-67.

36. The experiments recorded in Exhibit 5 were completed prior to December 23, 1997.

37. The experiments recorded in Exhibit 5 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

38. The experiments recorded in Exhibit 2, 4 and 5 therefore describe successful completion prior to December 23, 1997 of the construction of a chimeric DNA molecule (**pMBW233/239 series**) comprising in order

- a. a promoter operative in a eukaryotic (plant) cell (**Ubi-P**);
- b. a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (**Gusd in sense orientation**) in a eukaryotic cell; and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (**Gus5' in antisense orientation**);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (**Ubi intron**) (which is heterologous to the sense GUS sequence); and

- c. a DNA region involved in transcription termination and polyadenylation (**tml'**).

39. The experiments recorded in Exhibit 2, 4 and 5 also describe successful completion prior to December 23, 1997 of a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (**GUS gene**) in a eukaryotic cell (a plant cell, **rice**) comprising the step of introducing into the eukaryotic cell (plant cell) a chimeric DNA (**pMBW233/239 series**) as described in paragraph 24.

40. The statements made in this declaration are made on the personal knowledge or on the information and belief of the declarants. We hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1st May 2008

Peter Michael Waterhouse  
Peter Michael Waterhouse,

Date: 1st May 2008

Ming-Bow Wang  
Ming-Bow Wang

Date: \_\_\_\_\_

\_\_\_\_\_  
Michael Wayne Graham

Date: 1st MAY 2008

Neil A. Smith  
Neil A. Smith

Attorney's Docket No. 1021565-000060

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of	)	
	)	
Ming-Bo Wang et al.	)	Group Art Unit: 1635
	)	
Application No.: 09/287,632	)	Examiner: JANE J ZARA
	)	
Filed: April 7, 1999	)	Confirmation No.: 6526
	)	
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED PHENOTYPES	)	
	)	
	)	

**DECLARATION BY INVENTOR UNDER 37 C.F.R. § 1.131**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

We, Peter Michael Waterhouse, Ming-Bo Wang, Michael Wayne Graham and Neil A. Smith, citizens of Australia, hereby state as follows:

1. We are the named inventors of the subject matter of the above captioned application.

2. We have read and understood the Office Action dated November 1, 2007. We understand that U.S. Patent No. 6,506,559 has been cited in the Office Action in rejecting the claims of the present application.

3. We submit Exhibits 1-5 as evidence that embodiments of the presently claimed invention were made, i.e. actually reduced to practice, prior to the December 23, 1997 filing date of U.S. Provisional Application No. 60/068,562 of which benefit is claimed by U.S. Patent No. 6,506,559.

4. Exhibit 1 is a copy of 7 pages from a laboratory notebook prepared by Neil Smith that recorded experiments performed by Neil Smith and/or under his supervision during the construction of a chimeric DNA comprising two copies of a 0.75 kb fragment of the cDNA copy

**Buchanan Ingersoll & Rooney PC**  
Attorneys & Government Relations Professionals

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of the Potato Virus Y RNA genome (PVY) in inverted orientation under control of a CaMV 35S promoter and further comprising intron 2 of the *Pdk* gene from *Flaveria trinervia* in the transcribed region of the chimeric DNA.

5. Notebook page 151 (Exhibit 1, page 1) describes the relevant part of expression vector N2, which was used as starting material to produce the chimeric DNA as described in paragraph 4. That N2 is an expression vector can be seen from the notation ART7, which indicates the backbone of the expression vector pART7 which was commonly used within the CSIRO Plant Industry laboratories at that time. The figure indicates the restriction enzyme sites and denotes the different functional parts of the N2 vector as blocks. The bent arrows indicate the promoter orientation, while the straight arrows indicate the orientation of the PVY inserts. The sizes of the functional parts are indicated between brackets below the blocks and are noted as kilobasepairs. Abbreviations used are: 35S: Cauliflower Mosaic Virus 35S promoter; PVY: approximately 0.75kb fragment of the sequence encoding the protease of potato virus Y; S4: 0.6 kb fragment of the Subterranean Clover Stunt Virus segment S4; Ocs3': 3' termination and polyadenylation signal from the octopine synthase gene from *Agrobacterium tumefaciens*.

6. The notebook page 151 further indicates a first cloning strategy to produce a PVY hairpin RNA having complementary sense and antisense strands targeting the PVY genome part, whereby the 0.6kb HindIII fragment comprising the Subterranean Clover Stunt Virus segment S4 fragment was deleted. In the product, both copies of the PVY fragment are operably linked under the control of one CaMV35S promoter. Upon transcription from the CaMV35S promoter which is active in plant cells, a double stranded RNA molecule as schematically represented ("mRNA double stranded") would be produced. The RNA molecule as represented is an artificial hairpin RNA structure comprising two annealing RNA sequences labeled "sense strand" and " $\alpha$  sense strand", wherein one of the annealing RNA sequences comprises a sense sequence of 750 nucleotides identical to 750 consecutive nucleotides of a PVY target gene of interest in a eukaryotic cell, in this case a plant cell, and the second of the annealing RNA sequences comprises an antisense sequence identical to 750 consecutive nucleotides of the complement of the PVY target gene of interest.

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7. Notebook pages 186-188 (Exhibit 1, pages 2-4) describe the preparation of intron 2 of the *pdk* gene of *Flaveria trinervia* and cloning of a PCR amplified fragment having intron 2 (767bp) flanked by restriction enzymes into a small cloning vector (PBC) for amplification purposes.

8. On Notebook page 186 plasmid *pdk1* is described comprising a 10.5 kb *EcoRI* fragment of the *pdk* genomic DNA in pBLUESCRIPT vector. The figure is a schematic representation of the *pdkA* genomic DNA, the cloned *EcoRI* fragment and the position of intron 2. At the bottom is a photo of diagnostic restriction enzyme digests to confirm the structure of *pdk1*.

9. Notebook page 187 represents a nucleotide sequence alignment of the *pdkA* and *pdkB* genes with an indication of the position and nucleotide sequence of intron 2 of *pdkA*. Further indicated are the nucleotide sequences and position of the oligonucleotides used as PCR primers to specifically amplify the intron 2 fragment, flanked by *HindIII* restriction enzyme recognition sites.

10. Notebook page 188 details the PCR reaction protocol to amplify the intron 2 fragment from the plasmid *pdk1*, a gel analysis of the amplified fragment, a ligation protocol for introduction of the PCR- amplified, *HindIII* restricted product into a small vector named PBC and determination of the relative orientation of insertion of the *HindIII* fragment comprising the intron 2 by restriction analysis. At the bottom of the page, the two alternative orientations of insertion into PBC are schematically represented, together with an indication of the expected size of the fragment which was generated after *BglII*/*PstI* restriction digestion for both orientations and indication of the orientation of inserts in the different clones.

11. The first picture of the gel on Notebook page 189 concerns additional verification for the clones of the intron 2 *HindIII* fragment in PBC in both orientations. Immediately below the

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picture is a schematic representation of the cloning strategy to introduce the intron 2 between the two fragments of PVY in inverted orientation under control of a CaMV35S promoter, to generate a double stranded RNA-encoding chimeric gene. To this end, the 0.6 kb HindIII S4 fragment from T-DNA vector pNS2 was replaced by the 0.77 kb HindIII fragment comprising the intron 2 described under paragraph 10. pNS2 comprised the gene components of expression vector N2 described on notebook page 151 (discussed in paragraph 5 above) cloned into T-DNA vector pART27 (see Exhibit 1, page 6 where it is indicated that the backbone vector is pART27, a T-DNA vector commonly used at CSIRO for introduction of chimeric DNAs into plant cells by *Agrobacterium*-mediated transformation). Notebook page 189 further describes the composition of the ligation mix and the restriction enzyme analysis to identify clones in which the S4 fragment had been replaced by the intron 2 fragment. A small table at the bottom of notebook page 189 indicates the predicted sizes of fragments generated by restriction enzyme digests for each of the two possible orientations of the inserted intron fragment (indicated as "sense intron" and "α-sense intron", the latter is shorthand for intron in antisense orientation).

12. Notebook page 192 (Exhibit 1, page 6) displays the restriction enzyme analysis with *Apal* enzyme to verify which of the candidate clones contained a single insert of the intron fragment. As indicated next to the gel, the clones with a single insert were expected to produce a restriction fragment of 770 bp, and as stated there "appear to be many [candidate clones] with single Intron 2 inserts". The potentially good chimeric DNA constructs were further verified through *XbaI* restrictions enzyme analysis which as indicated were expected produce a characteristic 2.3 kb fragment. At least candidates "2 and 6 (from pool 7) appear to have [the] intron insert".

13. The bottom part of the Notebook page 192 then represents the restriction enzyme analysis with the restriction enzyme combinations indicated at the bottom of page 189 to determine the orientation of the intron 2 in the chimeric DNA constructs for 4 different clones. The notification under the gel pictures indicate that clone "2" had the intron in antisense orientation, while clones "6", "original single clone 1" and clone "6 from pool 7" had the intron fragment in the sense orientation. Intron 2 is a heterologous intron with respect to the PVY inserts in the chimeric DNA.



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14. Notebook page 193 (Exhibit 1, page 7) represents a quality control of a large scale DNA preparation of the T-DNA vector clones 2 and 6 from Notebook page 192. At the top of page 193 it is indicated that the chimeric DNA construct with the intron fragment in antisense orientation is named pNS9, while the chimeric DNA construct with the intron fragment in sense orientation is named pNS10. The bullet point "set up 2 and 6 for tri-parental => LBA4404" refers to the introduction of T-DNA vectors from the *E. coli* host into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid by a process referred to as triparental mating commonly used in the art. The resulting agrobacterial transconjugant strains were cocultivated with tobacco W38 cells in order to introduce the chimeric DNA into plant cells.

15. Exhibit 1 thus describes the successful construction of a chimeric DNA construct molecule comprising, in order:

- a) a promoter, operative in a eukaryotic (plant) cell (*CaMV35S*);
- b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the nucleotide sequence of the nucleic acid of interest (*0.75 kb PVY region in sense orientation- the nucleic acid of interest is thus comprised in the genome of an infecting RNA virus in this embodiment*)); and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*);wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequences; and

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wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and

- c) a DNA region involved in transcription termination and polyadenylation (3' *ocs* region).

16. The experiments recorded in Exhibit 1 were completed prior to December 23, 1997.

17. The experiments recorded in Exhibit 1 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

18. Exhibit 2 is a copy of 5 pages from a laboratory notebook (Notebook No 5) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision during the construction of a chimeric DNA encoding an RNA comprising sense and antisense nucleotide sequences targeted to a  $\beta$ -glucuronidase gene (GUS) in which the sense and antisense nucleotide sequences could basepair over about 558 bases, as described in the above mentioned US patent application in Example 1, Figure 1A (6) and Sequence Listing entry SEQ ID NO 1. Further recorded in Exhibit 2 are the introduction of the chimeric gene into a T-DNA vector and introduction of the resulting T-DNA vector into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid. The introduction of the chimeric DNA into plant cells, and data showing reduction of target gene expression in the plant cells are recorded in Exhibits 4 and 5.

19. Exhibit 2, page 1, entitled "To make invert-repeat GUS construct for rice transformation" recorded the starting materials and ligation mixes set up to introduce a second copy of part of the GUS coding region into a vector pWUJGdT comprising a GUS coding region with a deletion of the internal 0.3kb EcoRV fragment.

20. Two cloning strategies were followed i.e.

- a. to introduce a HincII fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 3) into EcoRI restricted vector pWUJGdT (indicated on top of the page as DNA 1 –

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treated to render the sticky ends of the restriction fragment blunt and further treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page. This ligation mix is recorded as ligation 2.

- b. To introduce a *Sma*I-EcoRV fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 4) into *Sma*I restricted vector pWUJGdT (indicated on top of the page as DNA 2 – treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page). This ligation mix is recorded as ligation 4.

21. Exhibit 2, page 2 recorded the identification of candidate clones resulting from the cloning strategies, as well as identification of the orientation of the insert into the vector. At the bottom of the page, these two possible insert orientations into the chimeric DNA are schematically represented, resulting in two different vectors namely:

- a. candidate clone 2 (2) renamed pMBW234 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the same orientation as the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml*').
- b. candidate clone 2 (4) renamed pMBW233 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the inverse orientation relative to the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml*'). Transcription of this chimeric gene yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, namely a sense RNA sequence identical to about 558 consecutive nucleotides of the GUS

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gene and an antisense RNA sequence identical to about 558 consecutive nucleotides of the complement of the GUS gene.

22. Exhibit 2, page 3 records the experiments performed to clone the chimeric genes described under paragraph 21, as NotI restriction fragments, into a T-DNA vector designated binary vector pWBVec4A. As indicated in the top right insert, "ligation 2" uses "DNA 1", i.e. NotI fragment from pMBW234 ; "ligation 3" uses "DNA 2" i.e. NotI fragment from pMBW233.

23. Exhibit 2, page 4 contains the continued recorded data from the experiments described under paragraph 22, including the restriction enzyme analysis, of candidate clones from ligations 2 and 3 mentioned on the previous page. The plasmid denoted pMBW237 refers to the chimeric gene in colony 2(8) [clone 8 of ligation mix 2, thus corresponding to a chimeric gene as in pMBW234], and the plasmid denoted pMBW239 refers to the chimeric gene in colony 3(8) [clone 8 of ligation mix 3, thus corresponding to a chimeric gene as in pMBW233].

24. At the bottom of Exhibit 2, page 4 it is indicated that T-DNA vectors 2(8) and 3(8) were used for triparental mating, referring to the introduction of the T-DNA vectors from the *E. coli* host into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid, and Exhibit 2, page 5 records the verification of the *Agrobacterium* transconjugants as containing the respective T-DNA vectors.

25. The experiments recorded in Exhibit 2 were completed prior to December 23, 1997.

26. The experiments recorded in Exhibit 2 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

27. Exhibit 3 is a copy of a publication by Alan H. Christensen and Peter H. Quail, entitled "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants", which was published in *Transgenic Research* Volume 5, 213-218 (May 1996). This publication describes the ubiquitin promoter region fragment used in the construction of the chimeric genes described in Exhibit 2, as explained under paragraph 21. As indicated in Figure 1, the Ubi promoter region included an intron (Ubi-1 intron) indicated in the Figure by an angled line) in the transcribed region. This publication is mentioned in the above mentioned patent application as a reference for the Ubiquitin promoter used in the chimeric constructs described in Example 1 of that patent

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application. At the time of the construction of the chimeric genes, described under paragraphs 20 and 21, we were aware of the presence of an intron in the transcribed DNA region under the control of the Ubi promoter region.

28. Exhibit 4 is a copy of 6 pages from a laboratory notebook (Notebook 96) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision to transform transgenic rice calli containing and expressing a recombinant GUS gene as a target gene of interest in a eukaryotic (plant) cell, with the T-DNA vectors described in Exhibit 2, and to analyse phenotypic expression of GUS in the supertransformed rice calli comprising the target gene and the chimeric DNAs. Data from these analyses correspond to the data recorded in the patent application as "Example 1".

29. Exhibit 4, page 1 indicates that calli were initiated from transgenic rice seeds 4R-V10-28 and 4R-V10-67, both expressing a recombinant GUS gene of interest integrated in the genome of the calli cells. This target gene is a transgene in the cells. The bottom half indicates that these calli were "supertransformed" using different Agrobacteria comprising the different T-DNA vectors including pMBW239 (experiment indicated as "7"). Note also the control experiments where an "empty" control T-DNA vector (pMBW223) was used (experiment 1) and where a conventional co-suppression construct (comprising a transcribed sense nucleotide sequence of the GUSd gene (pMBW225) was used for comparison of the level of phenotypic expression of the target gene (experiment 2).

30. Exhibit 4, page 2 recorded the data for Bialaphos resistant calli obtained with the different Agrobacteria described on the previous page (paragraph 24 above), indicating that such calli had been transformed by the T-DNA's including the chimeric DNA silencing constructs (or control constructs). The transgenic calli obtained by transformation of the initial V10-28 transgenic rice cells with pMBW239 are indicated as "7a" whereas the transgenic calli obtained by transformation of the initial V10-67 transgenic rice cells with pMBW239 are indicated as "7b".

31. Exhibit 4, page 2 at the bottom and continuing on page 3, recorded the data of an initial GUS staining experiment on parts of the transgenic calli that were obtained (V10-67 derived). In control experiment "1b" where the GUS transgenic calli were supertransformed by an "empty" T-DNA vector, all supertransformed calli strongly stained blue indicating strong GUS activity. On page 3 of Exhibit 4, the results of a similar GUS staining on calli obtained by supertransformation with pMBW239 indicating that the observed GUS staining was either weak

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("W") , negative ("-") or resulted in an isolated blue spot. These data indicated that the chimeric DNA from pMBW239 reduced the phenotypic expression of the GUS target gene in the rice cells.

32. Exhibit 4, pages 4 and 5 represent the recordation of these data for the individual calli. Exhibit 4, page 6 summarizes the data of an initial GUS staining experiment on parts of the obtained transgenic calli (V10-28 derived). While in series 1a, all calli transformed by the control T-DNA vector pMBW223 strongly stained blue indicating strong GUS activity, the transgenic calli obtained by transformation with pMBW239 (series 7a) all scored as "very weak" or "basically GUS negative" with the exception of only two calli. On the bottom of page 6, reference is made to (quantitative) GUS assays, the data of which are recorded in Notebook 5, starting on page 55, discussed hereinafter as Exhibit 5.

33. The experiments recorded in Exhibit 4 were completed prior to December 23, 1997.

34. The experiments recorded in Exhibit 4 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

35. Exhibit 5 is a copy of 17 pages from a laboratory notebook (Notebook 5) prepared by Ming Bo Wang that recorded quantitative GUS analysis experiments performed by Ming-Bo Wang and/or under his supervision, on the supertransformed rice calli described in Exhibit 4. Particular attention is drawn to the "raw data" concerning GUS analysis by kinetic value measurements on Exhibit 5, page 14 which are represented in Example 1, Table 2 of the above mentioned patent application. See e.g. data for Plate No 3, wells 1A to 2H which have in handwriting indications "7a1" to "7a14" referring to transgenic lines, which are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-28. Similarly, data for Plate No 3, wells 3A to 4D which have in handwriting indications "7b2" to "7b16" referring to transgenic lines are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-67.

36. The experiments recorded in Exhibit 5 were completed prior to December 23, 1997.

37. The experiments recorded in Exhibit 5 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

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38. The experiments recorded in Exhibit 2, 4 and 5 therefore describe successful completion prior to December 23, 1997 of the construction of a chimeric DNA molecule (**pMBW233/239 series**) comprising in order

- a. a promoter operative in a eukaryotic (plant) cell (**Ubi-P**);
- b. a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (**Gusd in sense orientation**) in a eukaryotic cell; and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (**Gus5' in antisense orientation**);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (**Ubi intron**) (which is heterologous to the sense GUS sequence); and

- c. a DNA region involved in transcription termination and polyadenylation (**tmi'**).

39. The experiments recorded in Exhibit 2, 4 and 5 also describe successful completion prior to December 23, 1997 of a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (**GUS gene**) in a eukaryotic cell (a plant cell, **rice**) comprising the step of introducing into the eukaryotic cell (plant cell) a chimeric DNA (**pMBW233/239 series**) as described in paragraph 24.

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40. The statements made in this declaration are made on the personal knowledge or on the information and belief of the declarants. We hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.


Date: \_\_\_\_\_

\_\_\_\_\_  
Peter Michael Waterhouse,

Date: \_\_\_\_\_

\_\_\_\_\_  
Ming-Bo Wang

Date: 2 May, 2008

  
\_\_\_\_\_  
Michael Wayne Graham

Date: \_\_\_\_\_

\_\_\_\_\_  
Neil A. Smith



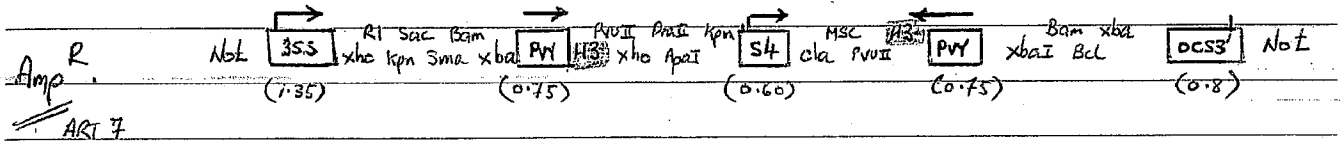
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WEDNESDAY

Date: .....

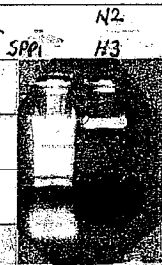
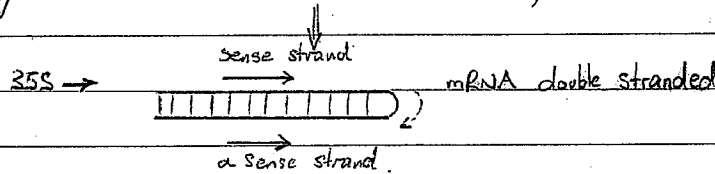
# CREATION OF PVY mRNA HAIRPIN ("DOUBLE STRANDED")

USING: N2 p 82 + 96



\* Cut 10 $\mu$ l of N2 with H3 o/n 37 $^{\circ}$ C

Will religate in 100 $\mu$ l to delete S4 promoter

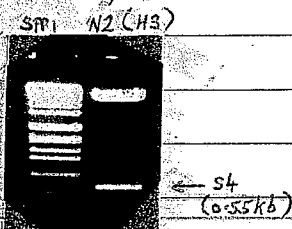


## LIGATION

VECTOR: 20 $\mu$ l digest RT.  
BUFFER: 2.0 $\mu$ l  
H<sub>2</sub>O: 62.0 $\mu$ l  
LIGASE: 3.0 $\mu$ l  
ATP(5mM): 5.0 $\mu$ l  
TOTAL: 100.0 $\mu$ l  
No colonies Rpt'd

Rpt. Cut 10 $\mu$ l N2 with H3 for 2 hours. (200 $\mu$ l Reaction)

Resuspended in 10 $\mu$ l dH<sub>2</sub>O. Ran lnd.



\* No clones - All still have insert!  
Will repeat by cutting band from  
TAE Agarose & ligating.

LIGATION: Rpt

Cut Plasmid: 9.0 $\mu$ l  
Buffer: 2.0 $\mu$ l  
dH<sub>2</sub>O: 7.0 $\mu$ l  
Ligase: 1.0 $\mu$ l  
ATP(5mM): 1.0 $\mu$ l  
TOTAL: 20.0 $\mu$ l

Signature of Researcher

Cont. PAGE 154

Date

2/1/01 DM52 cells JM109 cells

Signature of Supervisor

Def. Smith

Date

11/1/01

Experiment:.....

WEDNESDAY

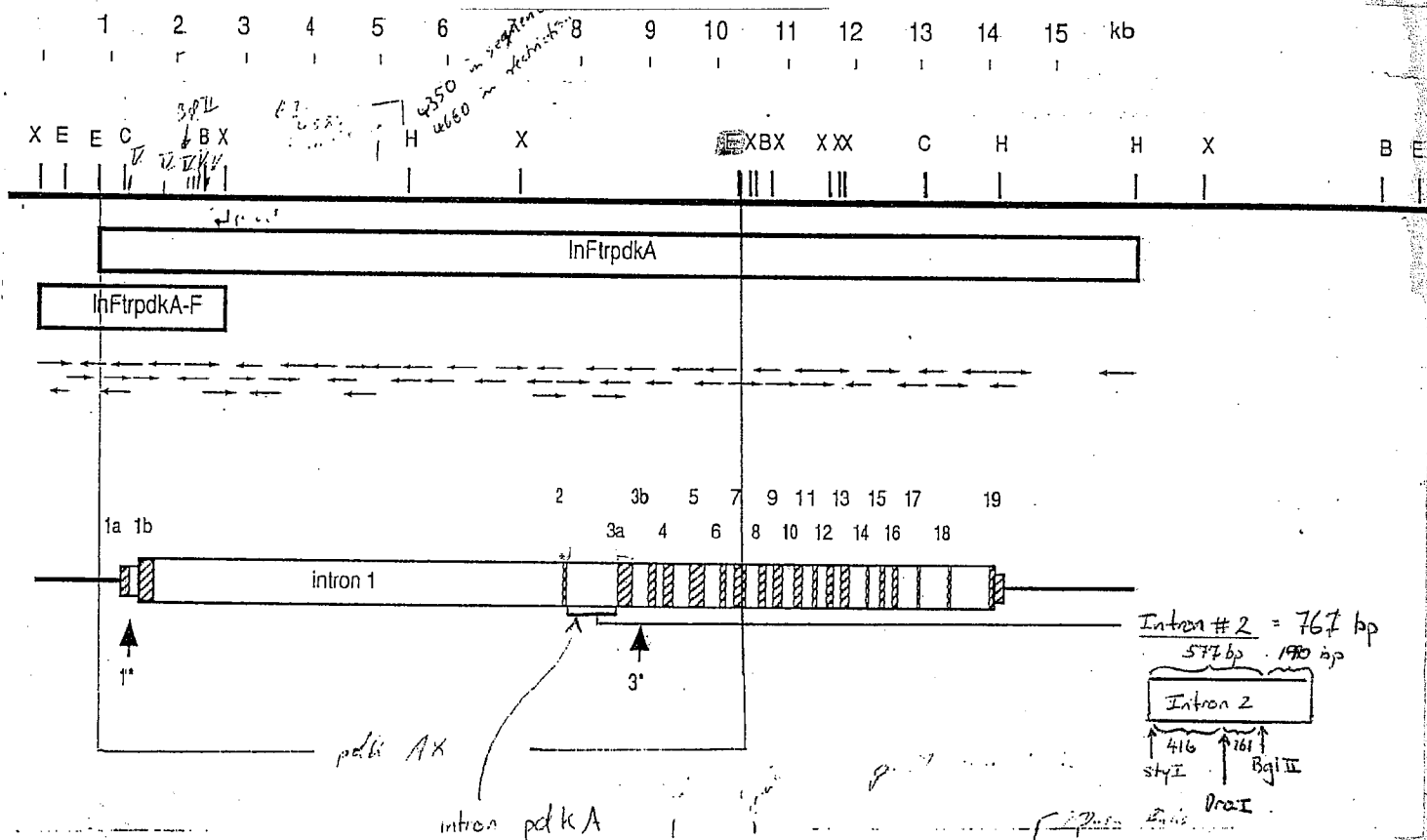
Date: .....

PREPARATION OF INTRON (2) FROM FLAVARIA travenaria

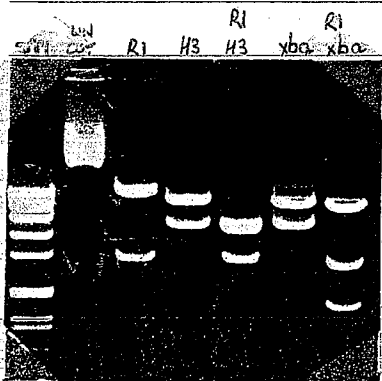
Plasmid provided by Bill Taylor

pdkA = *Flavaria travenaria*pdkB = *Flavaria brownii*

pdk1 - 13.75 kb plasmid

Intron is in pBluescript (ks) as an ~~ecoRI~~ fragment (10.5 kb) - T3-T7 orientat<sup>n</sup>

Set up Quagen prep + did diagnostic cuts



✓ R1 - 10.5 kb (insert) + 3.0 kb (Vector)

✓ H3 - 5.5 kb (5' end intron) + 7.5 (Rest)

✓ R1/H3 - " " 4.5 kb (3' end) + 3.0 kb (Vector)

✓ xba - 4-4.5 kb (centre)

R1/xba -

DNA STD (ng) QUAGEN (ul)  
200 150 100 50 0.1 0.25 0.5

= 200 ng / ul

W. Taylor



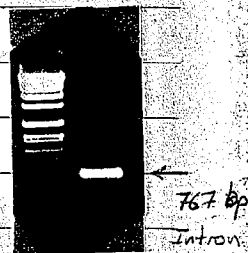
Experiment:

TUESDAY Date

## AMPLIFICATION OF Intron 2 from Pdk1

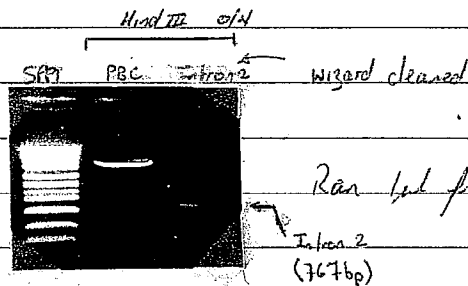
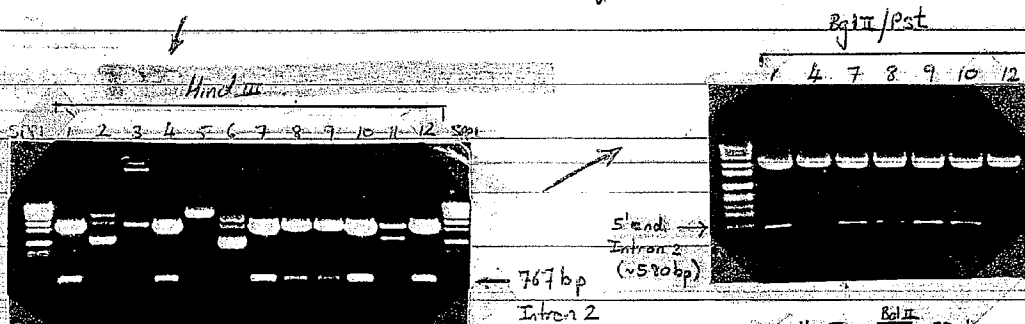
## PCR REACTION - Set up 2

DNA (20ng)	1.0 $\mu$ l	[ 95°C 2mins ] x 1
Amplifag BUFFER (x10)	1.0 $\mu$ l	[ 95°C 20sec, 56°C 30 sec, 72°C 1min ] x 30
MgCl <sub>2</sub> (25 mM)	1.0 $\mu$ l	[ 72°C 5 mins ] x 1
P.187 { 5' PRIMER (20ng)	1.0 $\mu$ l	* Did 2 identical reactions (10 $\mu$ l each)
3' PRIMER (20ng)	1.0 $\mu$ l	
dH <sub>2</sub> O	3.8 $\mu$ l	Ran 2.5 $\mu$ l of one reaction
2.5 mM dNTP <sup>3</sup>	1.0 $\mu$ l	* Pooled remainder, & prep etc
Amplifag Polym	0.2 $\mu$ l	2 $\mu$ l from 10 $\mu$ l
TOTAL	10.0 $\mu$ l	



## LIGATION Intron 2 → PBC

VACUOL (PBC:H3)	1.0 $\mu$ l	✓
INSER (Intron 2 PCR) H3	12.0 $\mu$ l	✓
BUFFER	2.0 $\mu$ l	✓
H <sub>2</sub> O	3.0 $\mu$ l	✓
LIGASE	1.0 $\mu$ l	
ATP (5mM)	1.0 $\mu$ l	✓
TOTAL	20.0 $\mu$ l	@ 25°C

Cut rest (8  $\mu$ l) in 200  $\mu$ l  
Reaction with Hind IIIcbl<sup>R</sup> B/W selection Cut with Bgl II / Pst → 577 bp or 190 bp

① PBC → kpa xho cla Sal H3 INT 2 H3 RV Pst sma ... 1,7,8,9,10

② PBC → kpa xho cla Sal H3 INT 2 H3 RV Pst sma ... 4,7,12

Na/Smith

Signature of Researcher

Signature of Supervisor

Hinc II (blunt)

Date

we Tard

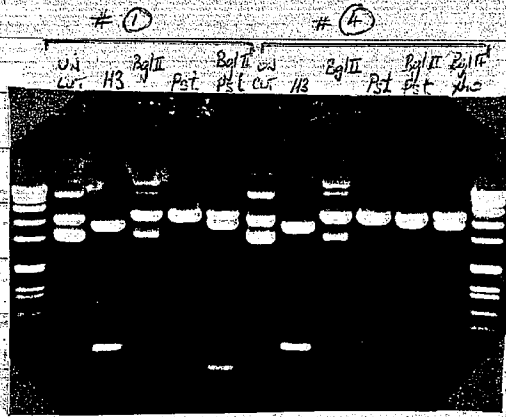
Na/Smith

Experiment:

Declaration 1  
Exhibit 1 Page 5  
US 09/287,632

Date

INTRON 2 IN PBC



#1 - In ori clon ① p 188  
#4 - In ori ② p 188

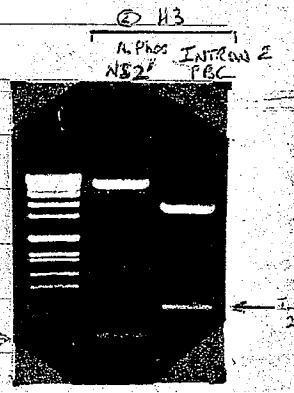
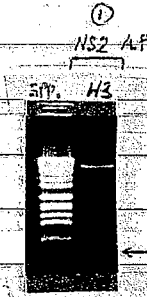
NS: BglII has only partially cut

\* All cuts are correct \*

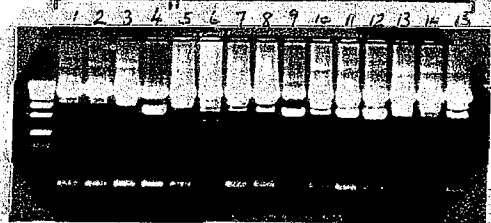
INTRON 2 → pNS2 (35S/PVY / PVY/OCSS')  
(cut w. H3)

SET UP 2 LIGATIONS ① & ②

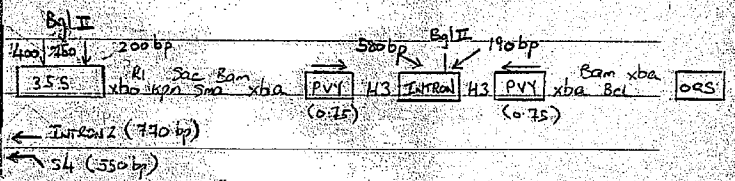
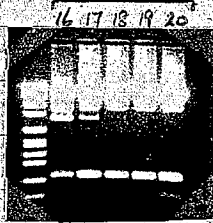
VECTOR (NS2-H3 A Phosph <sup>d</sup> )	① 5.0 μl	② 1.0 μl
INSERT (INTRON2/PBC H3)	✓ 5.0 μl	✓ 5.0 μl
BUFFER	✓ 2.0 μl	✓ 2.0 μl
H <sub>2</sub> O	✓ 6.0 μl	✓ 10.0 μl
LIGASE	✓ 1.0 μl	✓ 1.0 μl
ATP (5mM)	✓ 1.0 μl	✓ 1.0 μl
TOTAL	20.0 μl	20.0 μl



SINGLE COLONIES Pools x 6



SINGLE COLONIES Pools x 6



1.2 (H3 cut)

① xho H3  
cut H3 xho BglII BglII BglII



SENSE INTRON α-SENSE Intron  
✓ ① H3 → 770 bp (INTRON)  
✓ ② xho → Linearize  
✓ ③ BglII → 1.530 kb (35S' / PVY / INT 5') + 1.24 (35S' / PVY / INT 3') + 750 (from 35S)  
✓ ④ xho/BglII → 1.33 (PVY / INT 5') + 750 (35S3') + 900 (35S 3') + 940 (PVY / INT 3')  
✓ ⑤ H3/BglII → 950 bp (35S3' / PVY)  
✓ 580 (INT 5')  
✓ 190 (INT 3')

Signature of Supervisor

CONTINUED P 192

All correct in Handwritten

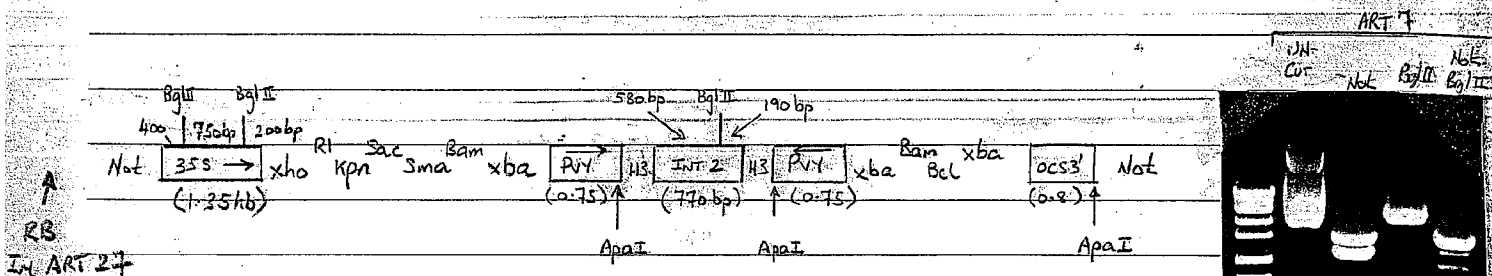
Date

W. T. P.

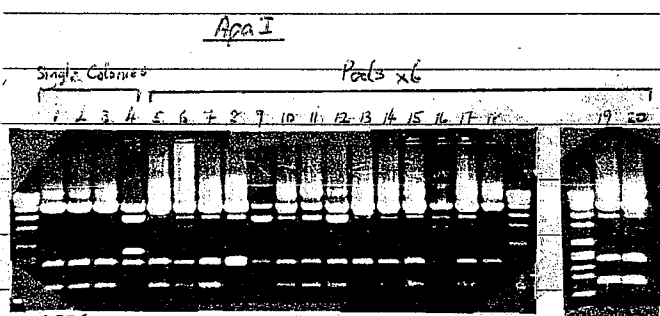
Experiment:

THURSDAY Date

Tubron 2 → PWS 2 (PWT HAIRPIN) CONT' P189



Cut Mini Preps Page 189 (20) with ApalI to confirm single inserts

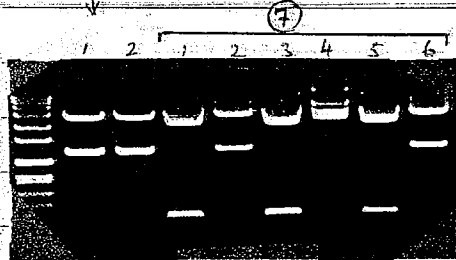


Expect

770 bp (Intron) +  
~1.5 kb (PWT/ocs3') (confirms with Cuts p. 189)

✓ Appear to be many with single Intron 2 inserts

Grow up single Colonies from #7  
+ cut with xbaI



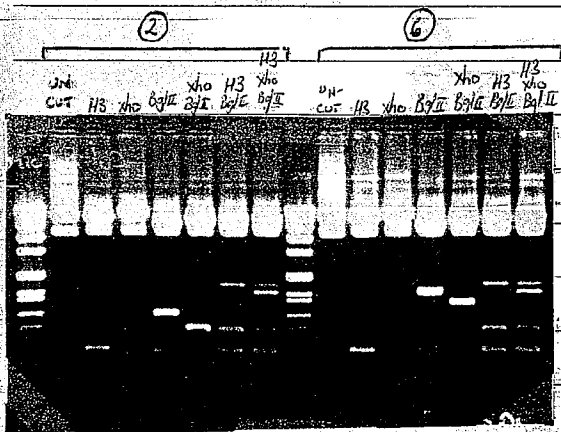
Expect

xbaI → ~2.3 kb (PWT/INT/PWT)

Again original 14.2 (p. 189)

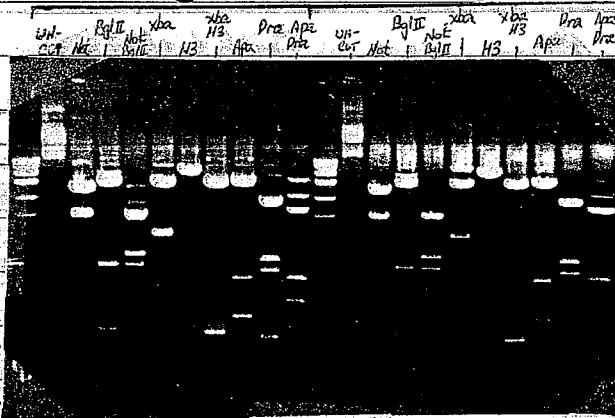
+ 2 &amp; 6 (from pool 7) appear to have intron insert

PWT deletion?  
due to Hairpin  
structure?



① from original  
single

⑥ (from pool 7)



INTRON IN

INTRON IN

Signature of Researcher

SENSE ORI

2-SENSE ORI

Signature of Supervisor

SET UP DIAGNOSIS OF ② &amp; ⑥

Date 9/6/01

Date 9/6/01

WCT

Naf Smith



Experiment: .....

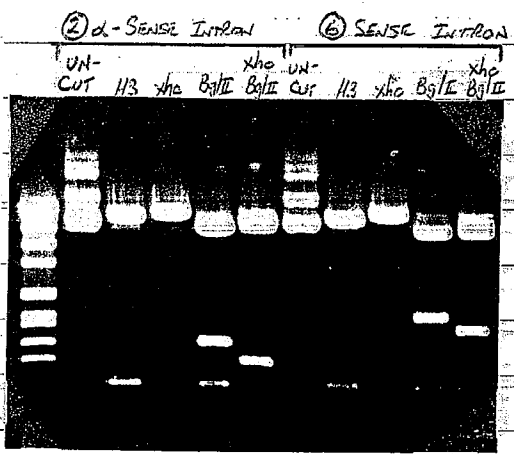
Declaration 1  
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Monday Date

CLONING DIAGNOSTIC CUTS



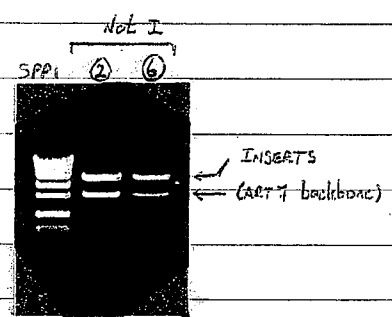
3 2-BglII  
1 35S



✓ All Cuts are correct

NOTED: ② α Sense Intron as pNS 9  
⑥ SENSE Intron as pNS 10

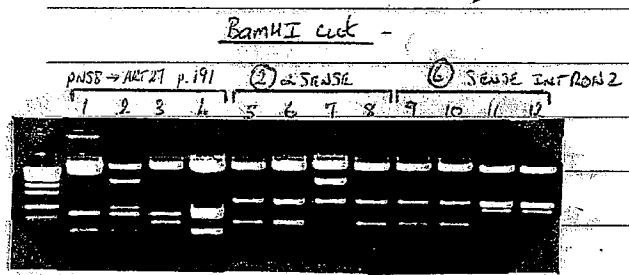
inserts



LIGATIONS FOR ② + ⑥ → ART 27

- ✓ VECTOR (ART 27 p.106) 1/20 : 1.0 μl
- ✓ INSERTS (② or ⑥ Not I) : 4.0 μl
- ✓ BUFFER : 2.0 μl @ R.T. o/h
- ✓ H<sub>2</sub>O : 11.0 μl
- ✓ LIGASE : 1.0 μl B/W Spec<sup>R</sup>.
- ✓ ATP (5mM) : 1.0 μl

insert

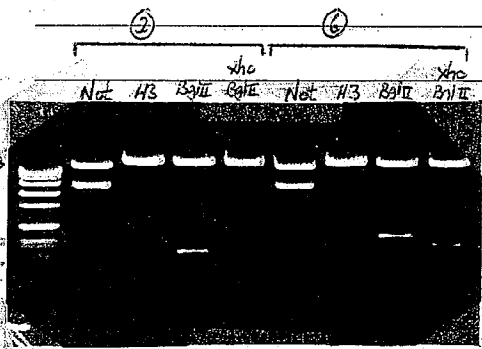


FOR ② + ⑥

✓ 35S at RB = 1.3 kb (ass's + sense of p17) + 2.0 kb (PVA / INT)

\* check # ⑧ + ⑨ FOR FURTHER CUTS

✓ MORE CUTS TO CONFIRM



\* SET UP ② + ⑥ FOR TRI-PARENTAL → LGA 4404

Expected

	NS8	NS9	NS10	Not	Expected
NS8	Not	xho	Not	xho	4.35 (INSAR)
					1.55 + 1.05 kb
NS9	Not	xho	Not	xho	4.40
					2.30
NS10	Not	xho	Not	xho	4.40
Date					2.30

Signature of Researcher

Signature of Supervisor

\* All Correct \*

Date

WCT  
Not

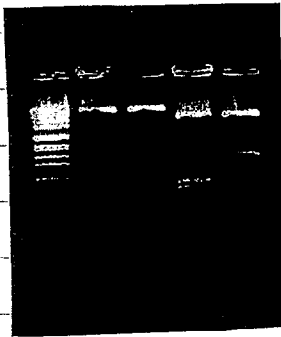
WCT

Project \_\_\_\_\_ (code) \_\_\_\_\_ Book No: \_\_\_\_\_ Cont'd from page \_\_\_\_\_

Experiment Title To make invert-repeat AUS construct for rice transformat

① } pWUJadT 2ul } pWUJadT 1ul } V: Purify ① with  
10x EcoRI 5 } 10x buffer 2 } phenol/chloroform exh. 5  
EcoRI 2.0 } SmaI 1.5 } treat with label kit  
H<sub>2</sub>O 41ul } H<sub>2</sub>O 15.5 } to blunt. Then purify  
with phenol/chloroform aces  
followed by AP treatment

③ } pWTKK-GUS 5ul } ④ } pWTKK-GUS 5ul } Δ: Treat ② with AP.  
10x buffer 3 5 } 10x buffer 4 5 }  
HincII 2 } SmaI\* 2 } \* SmaI added first.  
H<sub>2</sub>O 38 } EcoRV 2 }  
H<sub>2</sub>O 36 }



Run 4.5ul of each reaction (during digest  
or AP treatment)

Finally purify with phenol/chloroform.  
20ul DNA in H<sub>2</sub>O.

Ligation:

1. { DNA 1 2ul } 2. { DNA 1 2ul } 3. { DNA 2 2.0 } 4. { DNA 2 2.0 }  
10x lig 2 } DNA 3 3.0ul } 10x lig 2 } DNA 4 3.0 }  
ATP 1 } 10x lig 2 } ATP 1 } 10x lig 2 }  
Ligase 0.5 } ATP 1 } Ligase 0.5 } ATP 1 }  
H<sub>2</sub>O 14.5 } H<sub>2</sub>O 11.5 } H<sub>2</sub>O 14.5 } H<sub>2</sub>O 11.5 }

4°C,

Cont'd on page

Recorded by MING-BO WANG  
Print Name \_\_\_\_\_ Date \_\_\_\_\_  
Supervisor \_\_\_\_\_  
Read and Understood by \_\_\_\_\_  
Print Name \_\_\_\_\_ Date \_\_\_\_\_  
Signed \_\_\_\_\_ Initialled \_\_\_\_\_  
Signed \_\_\_\_\_



Project

(code)

Book No:

Cont'd from page

Experiment Title

Analyse colonies of lig 2 and 4 on page 25

37 colonies for lig 2

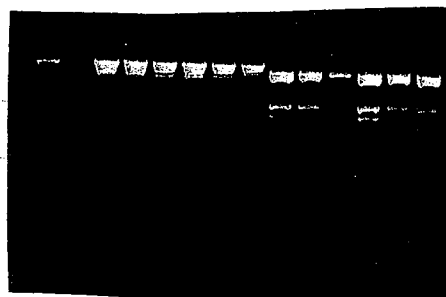
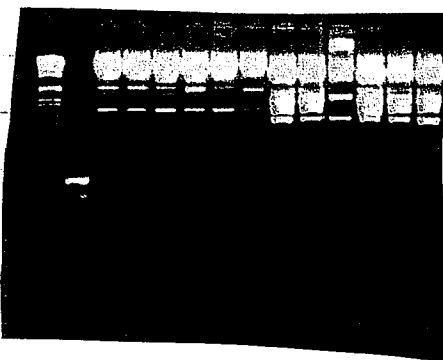
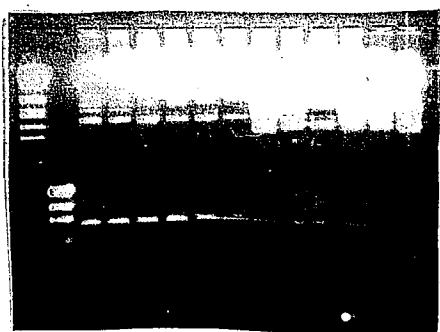
35 " " for lig 4

Pool the 72 colonies into 12 tubes. 60 µl DNA is TC

Digestion:

{	1, 2, 3, 4, 5, 6	3 µl
	10X buffer	2
	SnaBI	0.5
	SacI	0.5
	H <sub>2</sub> O	14 µl

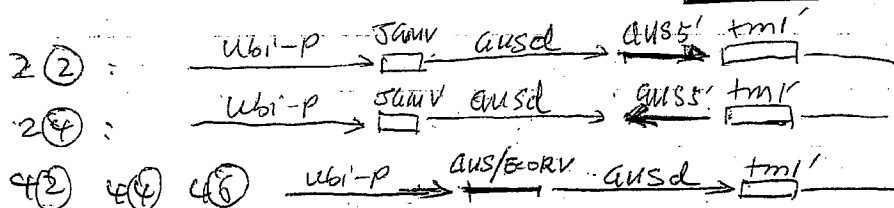
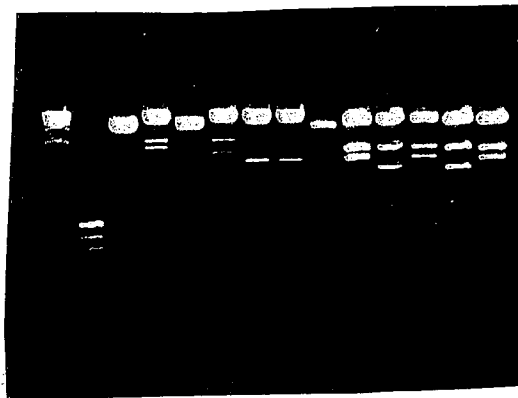
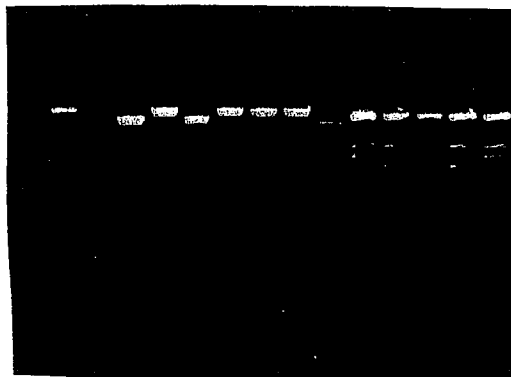
{	7, 8, 9, 10, 11, 12	
	10X E <sub>1</sub> W <sub>1</sub> I	
	NcoI	0.5
	EcoRI	0.5
	H <sub>2</sub> O	14 µl



Prepare DNA from separate colonies in pool 1 and pool 7.

60 µl DNA is TC.

Digest just as above.



pMBW234

pMBW233

orientation not checked

Cont'd on page pMBW234

Recorded by MING-BO WANG  
Print Name DateSupervisor  
DateRead and Understood by  
Print Name Date

Signed Ming Bo Wang

Initialled

Signed

MC TAG

ject

Experiment Title

Put pMBW232 and pMBW233 to binary pMBVecta

1. pMBW232 2.5  
10x buffer 3 5  
NotI 2  
H<sub>2</sub>O 40.5

2. pMBW233 2.5  
10x buffer 3 5  
NotI 2  
H<sub>2</sub>O 40.5

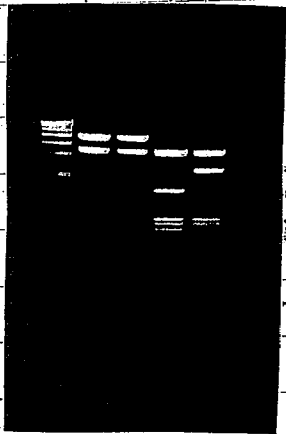
Ligations:  
1. pDNA1 ( ) 1.5  
10x lig 2  
ATP 1  
Ligase 0.5  
H<sub>2</sub>O 14.5

TO further check:

3. pMBW232 1  
10x buffer 4 2  
NcoI 0.5  
HincII 0.5  
H<sub>2</sub>O 16

4. pMBW233 1  
10x buffer 4 2  
NcoI 0.5  
HincII 0.5  
H<sub>2</sub>O 16

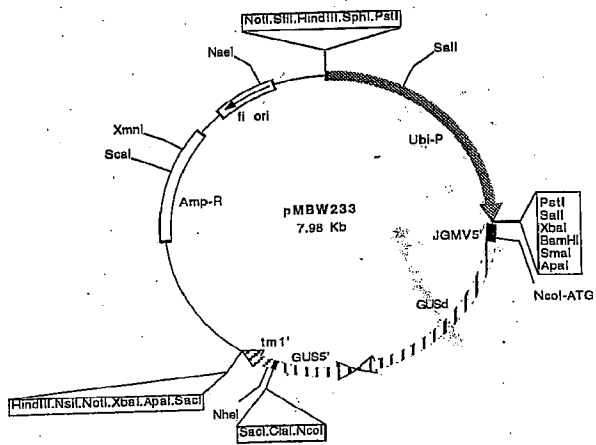
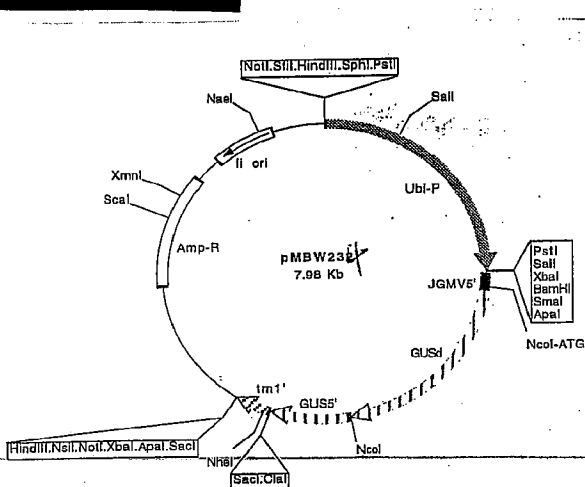
2. pDNA ( ) 1.5  
DNA1 ( ) 3  
10x lig 2  
ATP 0.5  
Ligase 0.5  
H<sub>2</sub>O 12  
3. pDNA ( ) 1.5  
DNA2 ( ) 3  
10x lig 2  
ATP 0.5  
Ligase 0.5  
H<sub>2</sub>O 12



Purify 1 and 2, 20µl DNA in H<sub>2</sub>O.  
Run 2µl each together with 3 and 4.

GUSd + GUS 5' (NcoI/HincII)  
GUSd  
ubi-P  
ubi-intron  
tm1' + GUS 5' (NcoI/HincII part.)  
→ tm1'

\* Both clones are correct.



Cont'd on page

Recorded by Print Name .....	Date .....	Supervisor Date .....	Read and Understood by Print Name .....	Date .....
Signed .....		Initialled .....		Signed .....

Project

(code)

Book No:

Cont'd from page

Experiment Title

Analyse colonies from Lys 2 and 3 of

30 µl DNA is TCR.

Digestion:

DNA 1.5 µl

20 20 20 25 26 = pMBW236

10x buffer 2

24 27 28 29 = pMBW237

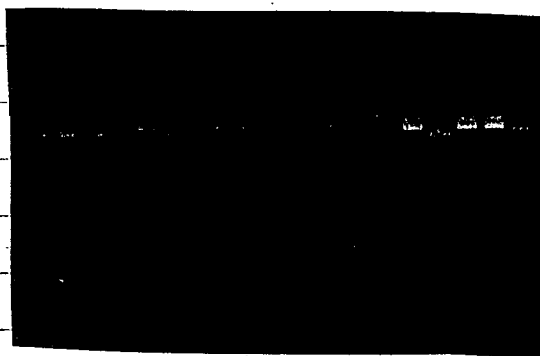
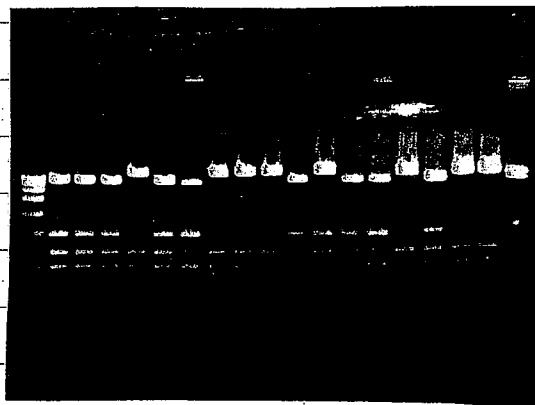
PstI 0.5

30 33 34 36 = pMBW238

H<sub>2</sub>O 16 µl

35 37 38 = pMBW239

32 and 39 did not look right.



Further check.

23 24 28 32 34 35 38 1.5 µl

10x buffer 3

2

NotI

0.5

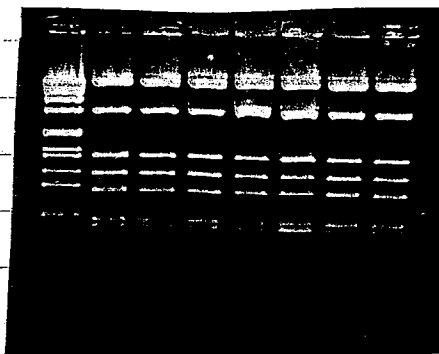
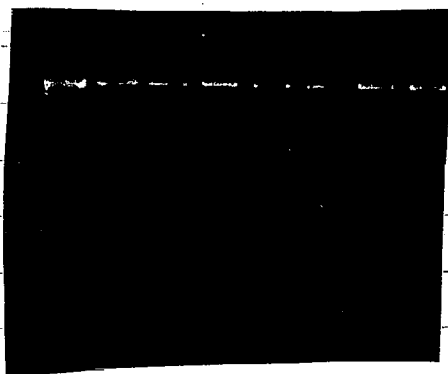
~~PstI~~

0.5

Salt

H<sub>2</sub>O

15.5



5' - CUSd - CUSd - tml  
ubi-p 3' part  
LB + tml + bar 3' half  
RB  
355 - bar 5' half  
RB or ubi-p 5'

28 and 38 were used for diploidal mating.

Cont'd on page

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Initialed .....

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Project

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Experiment Title

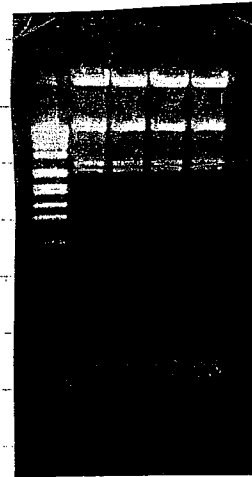
Analyse Agrobacterium dans conjugants.

1, 2 : AGLI = pmBW237 (28)

3, 4 : AGLI = pmBW239 (38)

Digestion:

DNA	5 µl
10X buffer	2
Pst I	0.5
H <sub>2</sub> O	12.5



check the TGMV vectors for Kpn I.

①	②	③	④	⑤
pwu5JT (N=2)	pwu5JICT (N=10)	pwu5JIFT (18)	pwu5JB:ET (22)	pwu5JBIFT (16)

10X buffer

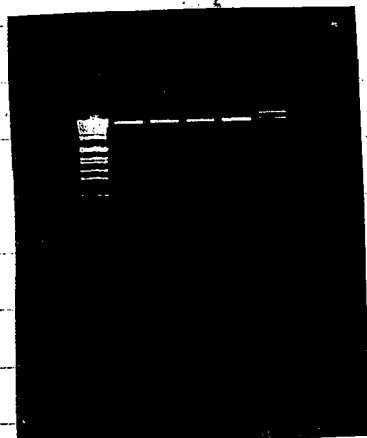
Kpn I

H<sub>2</sub>O

2

0.5

16.0



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Recorded by  
Print Name ..... Date .....

Supervisor  
Date .....

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Print Name ..... Date ...

Signed .....

Initialed

Signed .....

## TECHNICAL NOTE

# Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants

ALAN H. CHRISTENSEN<sup>‡</sup> and PETER H. QUAIL\*

Dept. of Plant Biology, University of California, Berkeley, CA 94720, and USDA/ARS Plant Gene Expression Center, 800 Buchanan St., Albany, CA 94710, USA (Fax: 1 510-559-5678)

Received 7 July 1995; revised 21 August 1995; accepted 24 August 1995

A set of plasmids has been constructed utilizing the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (*Ubi-1*) gene to drive expression of protein coding sequences of choice. Plasmids containing chimaeric genes for ubiquitin-luciferase (*Ubi-Luc*), ubiquitin- $\beta$ -glucuronidase (*Ubi-GUS*), and ubiquitin-phosphinothricin acetyl transferase (*Ubi-bar*) have been generated, as well as a construct containing chimaeric genes for both *Ubi-GUS* and *Ubi-bar* in a single plasmid. Another construct was generated to allow cloning of protein coding sequences of choice on *Bam*HI and *Bam*HI-compatible restriction fragments downstream of the *Ubi-1* gene fragment. Because the *Ubi-1* promoter has been shown to be highly active in monocots, these constructs may be useful for generating high-level gene expression of selectable markers to facilitate efficient transformation of monocots, to drive expression of reference reporter genes in studies of gene expression, and to provide expression of biotechnologically important protein products in transgenic plants.

**Keywords:** gene expression; transgenic monocots; ubiquitin

## Introduction

The general availability of strong promoters active in all or most cell types of monocotyledonous plants would be useful in a variety of applications in gene transfer studies with this plant group (McElroy and Brettell, 1994). Although the widely-used cauliflower mosaic virus (CaMV) 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells, and it is inactive in some cell types, e.g. pollen (Bruce *et al.*, 1989; Christensen *et al.*, 1992; McElroy and Brettell, 1994). The maize *Adh1* promoter has also been used in monocot transformation studies (Fromm *et al.*, 1990), but its activity appears to be restricted to root and shoot meristems, endosperm, and pollen (Kyojuka *et al.*, 1991). Because of their expected involvement in fundamental processes in all cell types, the genes for rice actin (*Act-1*) (McElroy *et al.*, 1990) and maize ubiquitin (*Ubi-1*) (Christensen *et al.*, 1992) have been investigated as

potentially useful alternatives to the CaMV 35S and *Adh1* sequences. Both of these monocot promoters have been shown to be significantly more active than the CaMV 35S promoter in monocot cells (Bruce *et al.*, 1989; McElroy *et al.*, 1990; Christensen *et al.*, 1992; Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; McElroy and Brettell, 1994) with the *Ubi-1* promoter being somewhat stronger than the *Act-1* promoter where compared directly (Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; Schledzewski and Mendel, 1994; Wilmink *et al.*, 1995).

Since our initial reports on the use of maize *Ubi-1* promoter constructs in transient (Christensen *et al.*, 1992) and stable (Toki *et al.*, 1992; Uchimiyu *et al.*, 1993) cereal transformation studies, we have distributed to a large number of researchers a variety of constructs with the *Ubi-1* promoter fused to a spectrum of selectable and scorable markers. Certain of these constructs or their derivatives have been used successfully in transforming a number of different monocot species (Wilmink *et al.*, 1995), including several cereals (McElroy and Brettell, 1994) and *Lemna* (Rolfe and Tobin, 1991), with reports of transgenic plants having been generated for rice (Cornejo *et al.*, 1993), wheat (Weeks *et al.*, 1993), and

\*To whom correspondence should be addressed.

<sup>‡</sup>Present address: Dept. of Biology, George Mason University, Fairfax, VA 22030, USA

barley (Wan and Lemaux, 1994). This report presents the structural details of the complete set of these constructs.

## Materials and methods

The cloning and sequencing of the maize ubiquitin gene and its promoter have been reported previously (Christensen *et al.*, 1992). All DNA cloning and manipulations reported here were performed according to standard protocols (Ausubel *et al.*, 1989). Restriction endonuclease digestions were carried out according to manufacturers' recommendations. GeneClean (BIO 101, La Jolla, CA, USA) was used to isolate specific restriction fragments from agarose gels. Recovery of DNA fragments was quantified by comparison of ethidium bromide fluorescence of an aliquot of the fragment with known masses of DNA on agarose gels. The *Hind*III linker (5' CAAGCTTG 3') used in the construction of pAHC27 was obtained from New England Biolabs. DNA ligations and subsequent transformations into competent *Escherichia coli* strain XL1-Blue or HB101 cells and plasmid DNA preparations were carried out using standard protocols (Ausubel *et al.*, 1989). Analysis of DNA sequences was performed using the UWGCG package of programs (Fromm *et al.*, 1990) and DNA Inspector II (Textco, W. Lebanon, NH, USA).

## Results

All of the constructs described here were generated by fusing the same 1992 bp *Pst*I fragment from the maize *Ubi-1* gene upstream of the relevant polylinker or marker sequence (Fig. 1). This *Ubi-1* *Pst*I fragment contains 899 bp of promoter sequence, 83 bp of 5' untranslated exon, and 1010 bp of first intron sequence, terminating through reconstitution of the *Pst*I site precisely at the G in the AG dinucleotide of the 3' splice junction of the intron (Christensen *et al.*, 1992). The nucleotide sequences at the fusion junctions at the 3' end of the *Ubi-1* DNA are shown for each construct in Fig. 2.

### pAHC17

This plasmid is a *Ubi-1* promoter expression vector for *Bam*HI (or *Bam*HI-compatible) cloning of protein coding regions. It contains the *Ubi-1* promoter, 5' untranslated region and intron upstream of an unique *Bam*HI site (Fig. 1). About 250-bp of nopaline synthase (NOS) 3' untranslated sequence and polyadenylation signals are located downstream of the *Bam*HI site. The 1992 bp *Pst*I fragment of the *Ubi-1* gene had been previously cloned into the *Pst*I site of M13mp19 for sequencing (Christensen *et al.*, 1992). A *Hind*III-*Bam*HI fragment from the replicative form of that clone was isolated and ligated to a 3175 bp *Hind*III-*Bam*HI fragment of pMF6 (Goff *et al.*, 1991) containing pUC8 sequence and 250 bp

of NOS 3' polyadenylation sequence adjacent to the *Eco*RI site.

The polylinker sequence is located between the end of the *Ubi-1* intron and the *Bam*HI cloning site and between the *Bam*HI site and the NOS sequence (Fig. 2). Thus, a reporter gene cloned into the *Bam*HI site is flanked by polylinker sequence on both the 5' and 3' sides. *Sal*I and *Xba*I sites from the M13mp19 polylinker are upstream of the *Bam*HI site and a *Sal*I and a *Pst*I site from the pUC8 polylinker are on the 3' side.

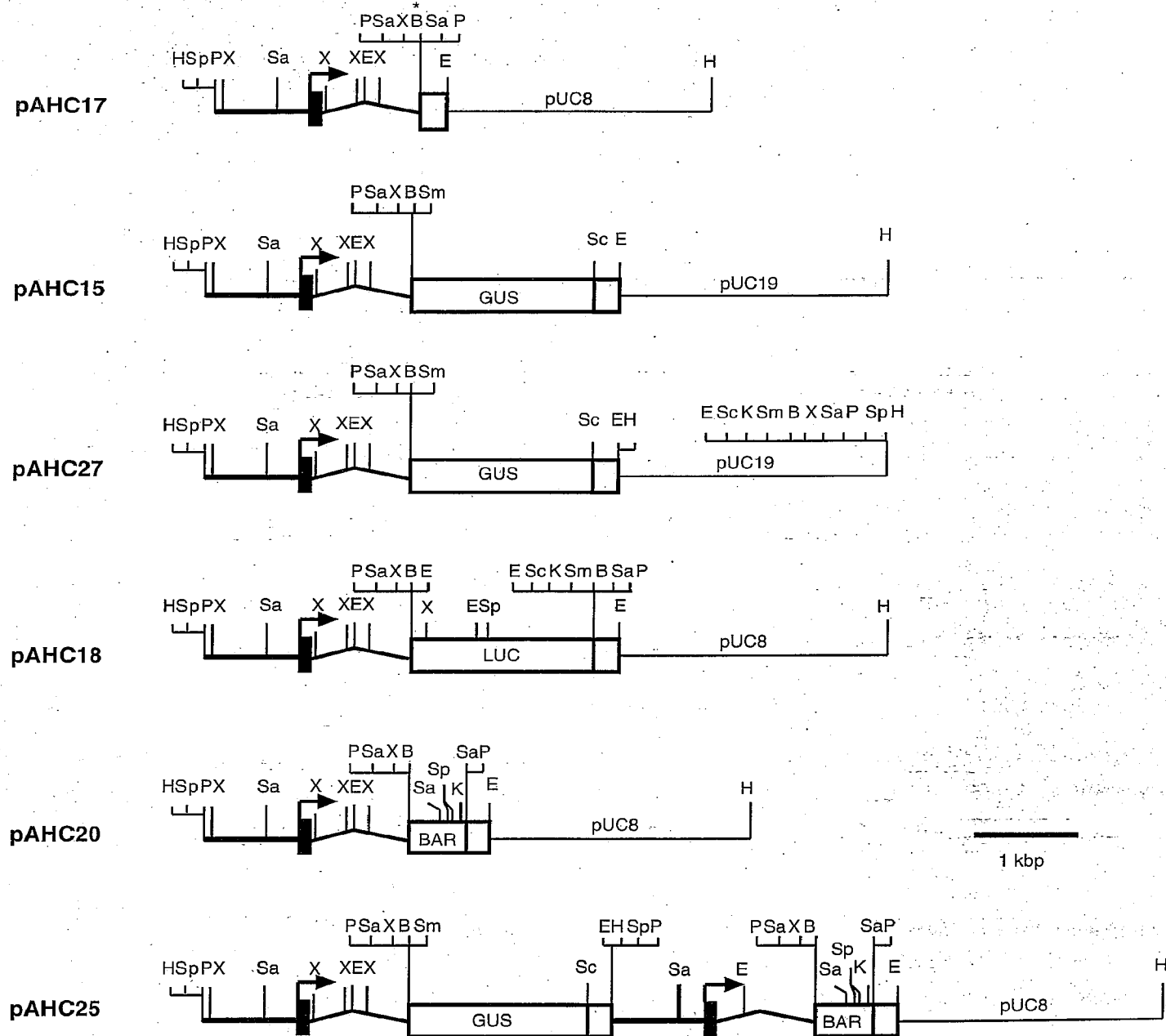
### pAHC15 and pAHC27 (pUbi-GUS)

These plasmids contain the maize *Ubi-1* promoter, 5' untranslated region and first intron fused to the coding region of the *E. coli uidA* gene (GUS) (Fig. 1). To produce pAHC15, *Hind*III-*Eco*RI fragment of pBI101.2 (Jefferson *et al.*, 1987) containing the *Hind*III to *Sma*I region of the pUC19 polylinker, the GUS coding sequence, and 260 bp of the nopaline synthase gene polyadenylation signal was cloned into the *Hind*III and *Eco*RI sites of pUC19 (pUC19-GUS-NOS). The 1992 bp *Pst*I fragment of the maize *Ubi-1* gene (Christensen *et al.*, 1992) was cloned into the *Pst*I site of the polylinker sequence upstream of the GUS coding sequence in pUC19-GUS-NOS. The construct contains the *Ubi-1* sequence in an orientation such that transcription will proceed through the ubiquitin 5' exon, intron and the GUS coding sequence, terminating in the NOS 3' sequence.

pAHC27 contains the same Ubi-GUS-NOS construct as pAHC15 but as a *Hind*III fragment cloned into the *Hind*III site of pUC19 (Fig. 1). This construct was generated to facilitate the production of pAHC25 (see below). The *Eco*RI site at the 3' end of the chimeric gene in pAHC15 is not unique as there is an additional *Eco*RI site in the *Ubi-1* intron. However, the *Hind*III site at the 5' end of the chimaeric gene is unique. To allow the entire construct to be removed as one fragment for further subcloning, a *Hind*III site was introduced at the 3' end of the chimaeric gene. This was achieved by partially digesting pAHC15 with *Eco*RI, optimizing the digestion for linear fragments. The *Eco*RI sites were filled in with dNTPs and Klenow fragment of DNA Polymerase and a *Hind*III linker (5' CAAGCTTG 3'; New England Biolabs) was added. Addition of the linker also restored the *Eco*RI site. The DNA was digested with *Hind*III to remove excess linker and to cut at the 5' end of the chimaeric gene. The 4.15 kb *Hind*III fragment containing the *Ubi-1 gus* chimaeric gene was gel-purified and subcloned into *Hind*III-digested pUC19. The chimaeric gene in the resultant pAHC27 is oriented such that the entire pUC19 polylinker is upstream of the *Ubi-1* promoter (Fig. 1).

### pAHC18 - pUbi-LUC

This plasmid contains the *Ubi-1* promoter-5' exon-first



**Fig. 1.** Schematic diagrams of expression vectors based on maize *Ubi-1* sequences. The relative sizes of the various segments of the linearized plasmids are drawn to scale. Bold straight line, *Ubi-1* promoter sequences; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; labelled open boxes, reporter gene sequences; blank open box, nopaline synthase 3' untranslated sequence; thin straight line, pUC8 (pAHC17, 18, 20, and 25) or pUC19 (pAHC15 and 27) sequence. Arrow at the *Ubi-1* exon signifies transcription start site and direction. GUS, β-glucuronidase (Jefferson *et al.*, 1987); LUC, firefly luciferase (Ow *et al.*, 1986); BAR, phosphinothricin acetyltransferase (De Block *et al.*, 1987). Restriction sites used in construction of the chimaeric genes and in adjacent polylinker sequences are shown. The *Bam* HI site marked with an asterisk in pAHC17 is a unique site for cloning *Bam* HI or *Bam* HI-compatible fragments. (Note: The *Xba* I sites located in the *Ubi-1* intron are subject to methylation interference in *dam*<sup>+</sup> *E. coli* strains. Also, although the *Eco* RI site in the *Ubi-1* intron upstream of the *gus* sequence in pAHC15 is cleaved efficiently, in both pAHC27 and pAHC25 the corresponding *Eco* RI site is cut very inefficiently). B, *Bam* HI; E, *Eco* RI; H, *Hin* dIII; K, *Kpn* I; P, *Pst* I; Sa, *Sal* I; Sc, *Sac* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xba* I.

**pAHC17****UBI1 Intron****NOS 3'**

Pst I Sal I Xba I Bam HI Sal I Pst I  
 .....ctgcagGTCGACTCTAGAGGATCCGTCGACCTGCAG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGCAGCTGGACGTC.....

**pAHC15 / 25 / 27****UBI1 Intron****GUS (from pBI101.2)**

Pst I Sal I Xba I Bam HI Sma I  
 .....ctgcagGTCGACTCTAGAGGATCCCGGGTAGTCAGTCCCTTATG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGGGCCCATCAGTCAGGGAATAC.....

**pAHC18****UBI1 Intron****LUC (from pDO432)**

Pst I Sal I Xba I Bam HI  
 .....ctgcagGTCGACTCTAGAGGATCCGAGCTTGGAAATTCCTTTGTGTACATTCTTGAATGTCGCTCGCAGTGACATTAGCATTCCGGTACTGTTGGTAAAAATG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGCTCGAACCTTAAGGAAACACAATGTAAGAATTACAGCGAGCGTCACTGTAATCGTAAGGCCATGACAACCATTTTAC.....

**pAHC20 / 25****UBI1 Intron****BAR (from pUC/BASTA)**

Pst I Sal I Xba I Bam HI  
 .....ctgcagGTCGACTCTAGAGGATCCATCGATTAGGAAGTAACCATG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGTAGCTAATCCTTCATTGGTAC.....

**Fig. 2.** Nucleotide sequence of the polylinker region comprising the junction between the *Ubi-1* intron and the reporter gene or NOS 3' sequence. The *Ubi-1* intron sequence (lower case) ends with a *Pst*I site containing the 3' splice junction. The reporter gene sequences shown downstream of the polylinker are those upstream of the respective coding sequence and end with the ATG translation start codon (italicized) shown for each.

intron fused to a luciferase (LUC) reporter coding sequence (Fig. 1). An 1892 bp *Bam*HI fragment of pDO432 (Ow *et al.*, 1986) containing 80 nucleotides of 5' untranslated sequence, the luciferase coding region (1649 nucleotides) and 163 bp of 3' untranslated sequence was cloned into the unique *Bam*HI site of pAHC17. This construct contains the luciferase coding sequence in the same orientation as the ubiquitin promoter.

**pAHC20 - pUbi-BAR**

The Ubi-BAR chimaeric gene in this plasmid provides selection of transformants resistant to Basta<sup>TM</sup> herbicide (phosphinothricin) (De Block *et al.*, 1987). The Ubi-BAR construct was formed by ligating a 570 bp *Bam*HI-*Bcl*I fragment containing the *bar* gene into the *Bam*HI site of pAHC17. The *bar* gene fragment was excised from a plasmid (pUC8/BASTA) obtained from Dr M. Fromm (Fromm *et al.*, 1990). The resultant pAHC20 plasmid has *bar* in the same orientation as the maize Ubi-1 promoter (Fig. 1). The construct contains 18 bp of sequence between the *Bam*HI site and the translation start codon

of the *bar* gene (Fig. 2). The *Bcl*I site is 11 bp downstream of the TGA stop codon.

The unique *Hin*dIII site at the 5' end of the Ubi-1 sequence makes this plasmid very adaptable. This restriction site is suitable for insertion of a second chimaeric gene, such as a scorable marker also driven by a second *Ubi-1* promoter, as detailed below for pAHC25, or for any other desired promoter-gene combination.

**pAHC25 - pUbi-GUS/Ubi-BAR**

pAHC25 contains both a selectable marker (*bar*) and a scorable marker (GUS), each under the transcriptional control of a separate *Ubi-1* promoter (Fig. 1). The two chimaeric genes were first assembled separately in pAHC20 and pAHC27 and then the double construct was formed. This was achieved by excising the Ubi-GUS-NOS-containing *Hin*dIII fragment from pAHC27 and subcloning it into *Hin*dIII-digested pAHC20. The resultant pAHC25 plasmid has both Ubi-BAR and Ubi-GUS chimaeric genes in the same orientation.



## Discussion

The high activity of the maize *Ubi-1* promoter has now been documented in transient and/or stable transformation configurations in a number of monocot systems including rice (Bruce *et al.*, 1989; Toki *et al.*, 1992; Cornejo *et al.*, 1993; Uchimiya *et al.*, 1993; Takimoto *et al.*, 1994), wheat (Taylor *et al.*, 1993; Weeks *et al.*, 1993), barley (Wan and Lemaux, 1994), sugarcane (Gallo-Meagher *et al.*, 1993; Taylor *et al.*, 1993), maize (Christensen *et al.*, 1992; Gallo-Meagher *et al.*, 1993) *Pennisetum* (Taylor *et al.*, 1993), *Panicum* (Taylor *et al.*, 1993) and *Lemna* (Rolfe and Tobin, 1991). Whether or not the high level of expression of selectable marker genes fused to *Ubi-1* actually increases the efficiency of recovery of fertile transgenic plants relative to less active promoters like that from the CaMV 35S gene is yet to be rigorously examined (see Wan and Lemaux, 1994). However, the high level of GUS expression provided by the *Ubi-GUS* constructs has proven valuable in enabling rapid histochemical screening of transformants for transgene activity (Cornejo *et al.*, 1993).

The original intron present in the 5'-untranslated region of the *Ubi-1* gene (Christensen *et al.*, 1992) was retained in all the constructs here because of numerous previous studies showing that introns frequently strongly enhance transgene expression in cereals (Callis *et al.*, 1988; Bruce and Quail, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1993). The influence of the *Ubi-1*-intron has not been tested directly, but there is evidence that this maize sequence is spliced correctly in transgenic rice cells (Toki *et al.*, 1992).

Detailed examination of the spatial and temporal expression patterns of the *Ubi-1* promoter in transgenic plants is yet to be reported. However, initial data with a *Ubi-gus* construct indicate expression in all organs of transgenic rice consistent with a potential for targeting a wide spectrum of cells (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994).

An additional potentially useful feature of the *Ubi-1* promoter is that it is stress-inducible. Both thermal and mechanical stress have been shown to cause a strong enhancement of the *Ubi-gus* transgene activity in transformed rice (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994). It is possible that this fact may result in stronger expression of selectable marker fusion genes during the early stages of transformation, where recipient cells are exposed to a variety of stresses such as high osmotic pressures, particle bombardment and growth on toxic compounds. A subsequent decrease in expression level is expected upon removal of the selective conditions so that regenerated transgenic plants would presumably not continue to express the marker at high levels when it is no longer needed. The stress-inducibility of the *Ubi-1* promoter might also be useful for driving conditional

expression of genes that confer tolerance or resistance to various biotic and abiotic stresses such as pathogen attack, heat and water deficit (Takimoto *et al.*, 1994).

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Initiate calli from transgenic ~~plant~~ seeds (from To plants of 4R-VIO-28 and 4R-VIO-67).

De-husk, sterilize with ethanol for ~ 1 min, then 20% bleach ~~for~~ for ~ 25 min. Wash 4 times with H<sub>2</sub>O. place on NB. keep at 27°C in darkness.

Stain the tips of shoots for GUS:

VIO-28: 150 total, 32 GUS-negative

VIO-67: 108 total, 25 GUS-negative.

cut the scutellum, and place on fresh NB medium. keep at 27°C.

transformation:

same procedure as before. Use 10 ml NB-AS to wash off 2-day old bacterium from each plate (LB-RS). The calli were from 2 to 3 subcultures. GUS expression is good for both VIO-28 and VIO-67. Separate the two lines for the experiments.

Constructs:

AGL2: pMBL223	-	①	Ubi <sup>+</sup> -tm1'
" : " 225	-	②	Ubi <sup>+</sup> -JAMV-GUSd-tm1' (NO STOP)
" : " 227	-	③	Ubi <sup>+</sup> -JAMV-JAMV-GUSd-tm1'
" : " 229	-	④	Ubi <sup>+</sup> -JAMV-JAMV-iCAT-GUSd-tm1'
" : " 231	-	⑤	Ubi <sup>+</sup> -JAMV-JAMV-iCAT-GUSd(AS)-tm1'
" : " 237	-	⑥	Ubi <sup>+</sup> -JAMV-GUSd-GUSs(AS)-tm1'
" : " 239	-	⑦	Ubi <sup>+</sup> -JAMV-GUSd-GUSs'(AS)-tm1'

Wash the calli with H<sub>2</sub>O-Timentin twice, blot on filter paper, then transfer to NB-Timentin150-Hygromycin50-Protaphos5. Keep at 27°C.

Signature of Researcher

Wing Way

Date

Signature of Supervisor

W. Tang

Date

Experiment:

Date: / /

Transfer all calli to NB-Timentin 150 - hygromycin 50 - bialaphos 10 mg/L.

Transfer all calli to NB-Timentin 150 - hygromycin 25 - bialaphos 10 mg/L.

1. Transfer resistant callus pieces to NB-Timentin 150 - hygromycin 25 - bialaphos 10 mg/L.

Constructs/lines	Total number of callus pieces	No of bialaphos resistant callus.
1a: pMBW223/V10-28	115	25
1b: pMBW223/V10-67	71	36
2a: pMBW225/V10-28	124	26
2b: pMBW225/V10-67	63	25
3a: pMBW227/V10-28	122	20
3b: pMBW227/V10-67	77	22
4a: pMBW229/V10-28	<del>86</del> 113	30
4b: pMBW229/V10-67	73	24
5a: pMBW231/V10-28	161	22
5b: pMBW231/V10-67	88	36
6a: pMBW237/V10-28	125	21
6b: pMBW237/V10-67	79	21
7a: pMBW239/V10-28	193	28
7b: pMBW239/V10-67	176	23

For these lines which ~~had~~ proliferated well and produced relatively more number of callus pieces transfer part of the callus pieces to NB-TURO B5, and keep part of them on the old plates. Also stain some <sup>of V10-67-derived</sup> for assay. Look promising.

1b: lines 1, 2, 3, 5, 7, 8, 10, 11, 12, 13, 15, 17, 19, 23, 25. All are <sup>⊕</sup> strong.

2b: lines 4, 5, 10, 11, 14. 10 not blue; 4, 14 weak; 5, 11 strong.

3b: <sup>W+S</sup> 1, 3, 5, 6, 7, 8, 9, 10, 16, 18. S - strong; ⊕ - negative; W - weak; W+S - A piece is strong, the rest is weak.

⊕ - blue spot.

Signature of Researcher Khyas Waj 2

Date

Signature of Supervisor MC Tay

Date

Experiment:.....

Date / /

4b: lines:

(3) (4) (5) (7) (8) (9) (12) (14) (16) (17) A blue spot

5b: lines

(3) (4) (8) (9) (14) (17) (19) (23) (24) (25) A blue spot

6b: lines

(2) (4) (6) (8) (9) (10) (11) (13) (14) (15) very weak s+w

7b: lines

(2) (3) (5) (6) (10) (13) (14) (15) A blue spot  
A relatively big blue spot.  
callus missing

Transfer some good callus piece onto PRT15H25B5.

Transfer remaining good callus pieces into PRT15H25B5 or PRT15H25B5.

Note: The medium missed glutamine.

Transfer all the calli transferred to PRT15H25B5 on to RTH20B5. Keep under light at 24°C.

Stain the callus pieces for GUS. — see result next attached page.

Some pieces also stained on — Scan file  
plate.tif

GUS-negative or weak lines:

2a: 3, 7, 10, 12  
blue spot

3a?: 2, 4 (one blue piece one white piece)

4a?: 5, 7

5a: none.

6b: 4, 6, 8, 9, 12

6a: 1, 3

7b: 5, 3, 6

7a: 4, 8, 9

2b: 1, 10, 12

3b: 1, 2, 3, 12, 18

4b: 7, 8, 11, 12, 16, 17

5b: none

Signature of Researcher

Date

Signature of Supervisor

Date

scan file: plat01.tif

1a.	1	2	3	4	5	7	5	15	1	2	3	4
	S	S	S	S	S	S	S	S	S	S	S	S
	1	2	3	4	5	7	5	15	1	2	3	4
	S	S	S	S	S	S	S	S	S	S	S	S
1b	5	6	7	8	9	10	11	12	13	14	15	16
	S	S	S	S	S	S	S	S	S	S	S	S
1c	18	19	20	21	22	23	24	25				
	S	S	S	S	S	S	S	S	S	S	S	S
2a	1	2	3	4	5	6	7	8	9	10	11	12
	S	S	S	S	S	S	S	S	S	S	S	S
2	14	15	16	17	18	19	20	21	22	23	24	25
	S	S	S	S	S	S	S	S	S	S	S	S
3a?	1	2	3	4	5	6	7	8	9	10	11	12
	S	S	S	S	S	S	S	S	S	S	S	S
4a?	4	5	6	7	8	9	10	11	12	13	14	15
	S	S	S	S	S	S	S	S	S	S	S	S

S = strong  
M = medium  
Θ = negative  
W = weak

Scan file: platb2.tif

plate (II)

4a?	4b	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
4a?	4b	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

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plate (III)

7a.	7b.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
7a.	7b.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100



179

Experiment

Date / /

Transfer all good calli from PRTH-B5 to ATHT-B5.

Stain the following for GUS:

- 1a → 1, 2, 3, 4, 5, 7, A 1b → 1, 5, 7, 10, 11  
12, 16, 17, 19, 23, 25 — All blue
- 2a → 1, 4\*, 5, 8, 10, 12, 13, 14, 15, 16, 18, 19  
10, 4, 5, 12, 19 — very weak or GUS-
- 3a? → 1, 2, 3, 4, 6, 7, 8, 3b, 1, 2, 3, 16, 18
- 4a? → 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 4b, 8, 10  
11, 12, 13, 14, 15, 17
- 5a? → 1, 4, 6, 8, 9, 10, 12, 15, 16, 17, A
- 6a → 1, 3, 4, A, 6b → 4, 5, 9, 10, 11, 13, 14  
15, 16
- 7a → 2, 4, 6, 7, 8, 9, 10, 11, A, 7b → 2, 5, 6  
10, 11, 13, 14, 15

○ — very weak GUS

○ — basically GUS-

Stain ~~all~~ callus pieces from all lines on regeneration media. These are all the callus lines obtained. Keep the rest for regeneration.

GUS assay — see page 55, book 5.

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Experiment Title

GUS Assay — super-transformed rice calli

Materials: choose healthy callus pieces from each line (normally a bit green) put into <sup>2x</sup>Eppendorf. Keep one Eppendorf at RT for protein extraction. Keep the other in  $-70^{\circ}\text{C}$  freezer for DNA & RNA isolations. Also place a few smaller callus pieces from ~~most~~ most of the lines in NB medium for proliferation. All the calli were on regeneration medium (for a few weeks). See page 178 of book 4 for more details about the callus materials.

Protein Extraction = Follow the method on page 61 of the Thesis. Weigh the calli, add equal amount of the extraction buffer to each Eppendorf (i.e. 100  $\mu\text{g}$  to 100  $\mu\text{m}$ ). Grind with the machine for  $\sim 10$  sec at  $\sim 200$  rpm. Keep on ice first and then at  $-70^{\circ}\text{C}$ . The next day thaw the slurry and spin twice and collect the supernatant. ~~Keep~~ Store at  $-70^{\circ}\text{C}$ .

Protein Quantification:

Add 100  $\mu\text{L}$  H<sub>2</sub>O to a cuvette, then add 1  $\mu\text{L}$  of the sample, followed by 200  $\mu\text{L}$  BioRAD ~~reagent~~ reagent solution. Mix and measure the OD at 595 nm. make a standard curve using BSA.

Results: see the attached pages.

GUS Assay = Follow the method in the thesis: add

9  $\mu\text{L}$  extraction buffer, 2  $\mu\text{L}$  sample and 10  $\mu\text{L}$  reaction buffer to each well on the microtiter plate. Incubate at  $37^{\circ}\text{C}$  for  $\sim 40$  min. Stop the reaction by adding 180  $\mu\text{L}$  0.2 M Na<sub>2</sub>CO<sub>3</sub> to each well. Measure with FluoroScan. The reaction solution & 4.2 mg MUG in 10 ml extraction buffer.

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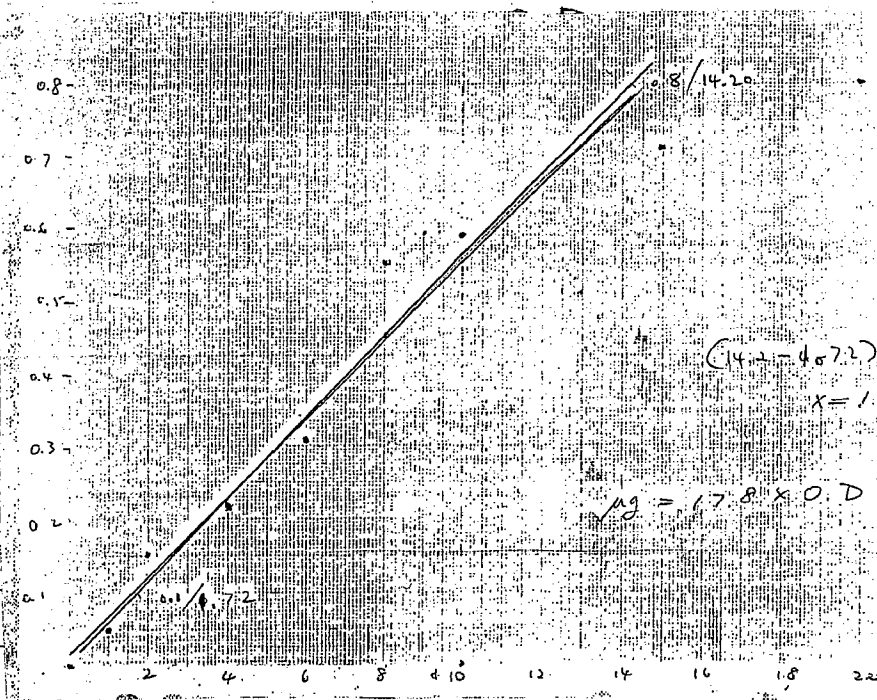
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Experiment Title

BSA	0.0.
1 ml -	.09
2 ml -	.15
4 ml -	.22
6 ml -	.31
8 ml -	.45
10 ml -	.59
15 ml -	.71
20 ml -	.80
25 ml -	1.02



		00	concentration		
1a1 -	.49	8.7	10/612 -	.56	10.0
1a2 -	.53	9.4	11/613 -	.54	9.6
1a3 -	.55	9.8	12/614 -	.48	8.5
1a4 -	.53	9.4	1/615 -	.44	7.8
1a5 -	.52	9.2	2/616 -	.40	7.1
1a6 -	.42	7.5	3/617 -	.47	8.4
1a7 -	.53	9.4	4/618 -	.49	8.7
1aA -	.59	10.5	5/619 -	.49	8.7
1aB -	.56	10.0	6/620 -	.45	8.0
1aC -	.61	10.8	7/620* -	.51	9.1
1b1 -	.29	5.2	8/623 -	.37	6.6
1b2 -	.41	7.3	9/624 -	.43	7.6
1/63 -	.21	3.7	10/625 -	.46	8.2
2/64 -	.36	6.4	11/2a1 -	.24	4.3
3/65 -	.31	5.5	12/2a2 -	.30	5.3
4/66 -	.49	8.7	1/2a3 -	.41	7.3
5/67 -	.27	4.8	2/2a4 -	.56	10.0
6/68 -	.46	8.2			

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	9/611 -	32	5.7		

## Experiment

3205- .53 9.4	11302- .25 4.4
4206- .44 7.8	12303- .18 3.2
5207- .55 9.8	1304- .18 3.2
6208- .65 11.6	2305- .21 3.7
7209- .41 7.3	3306- .25 4.4
82010- .51 9.1	4307- .26 4.6
42011- .50 8.9	5308- .19 3.4
02012- .32 5.7	6309- .26 4.6
12013- .30 5.3	730A- .28 6.8
13014- .36 6.4	8301- .34 6.0
2015- .33 5.9	9302- .55 9.8
22016- .44 7.8	10303- .31 5.5
32017- .29 5.2	11305- .32 5.7
42018- .32 5.7	12306- .35 6.2
520A- .62 11.0	1307- .42 7.5
6201- .33 5.9	2308- .29 5.2
7202- .32 5.7	3309- .23 4.1
8203- .47 8.4	43010- .26 4.6
9204- .54 9.6	53011- .31 5.5
10205- .52 9.2	63012- .32 4.3
11206- .57 10.1	73014- .33 5.9
1207- .44 7.8	83016- .62 11.0
1309- .24 4.3	93017- .55 9.8
12010- .27 4.8	103018- .29 5.2
22011- .39 6.9	11401- .32 5.7
42012- .21 3.7	12402- .22 3.9
52014- .41 7.3	1403- .42 7.5
62016- .27 4.8	2404- .31 5.5
72017- .31 5.5	3405- .31 5.5
82019- .31 5.5	4406- .32 5.7
920A- .24 4.3	5407- .29 5.2
10301- .18 3.2	6208- .36 6.4

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Experiment Title

First Reading of the Gas Assay.

ite No 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	14934	14754	14783	14722	14669	14980	14311	14951	14855	14794	15286	14587
B	15325	14732	14841	14719	15070	14584	14476	14564	14648	14752	14772	14639
C	16189	16166	1617	1618	1618	1620	1620	1622	1624	1625	1621	1622
D	203	204	205	206	207	208	209	210	211	212	213	214
E	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026
F	259	260	261	262	263	264	265	266	267	268	269	270
G	3014	305	306	307	308	309	310	311	312	313	314	315
H	367	368	369	370	371	372	373	374	375	376	377	378

ite No 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	400	401	402	403	404	405	406	407	408	409	410	411
B	400	401	402	403	404	405	406	407	408	409	410	411
C	400	401	402	403	404	405	406	407	408	409	410	411
D	400	401	402	403	404	405	406	407	408	409	410	411
E	400	401	402	403	404	405	406	407	408	409	410	411
F	400	401	402	403	404	405	406	407	408	409	410	411
G	400	401	402	403	404	405	406	407	408	409	410	411
H	400	401	402	403	404	405	406	407	408	409	410	411

TRANSF. 4:

ite No 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	273.2	283.9	273.2	240.4	23.20	0.094	0.094	0.204	0.408	0.173	0.141	0.377
B	88.46	116.3	718.15	218.1	0.173	0.188	0.566	0.157	0.157	0.188	0.110	
C	147.5	97.77	763	46.28	134.9	0.188	0.204	0.283	0.833	0.314	0.518	0.126
D	46.48	61.26	765	121.1	78.18	0.220	0.267	0.220	0.314	0.236	0.440	0.534
E	384.9	484.0	766	207.5	14.00	0.157	0.314	0.550	0.283	0.393	0.141	0.141
F	410.4	503.1	767	128.8	12.35	0.126	0.094	0.283	0.408	0.660	3.236	0.188
G	999.4	36.74	768	31.40	9.033	0.157	0.314	0.503	0.440	0.110	0.094	0.126
H	525.7	32.17	769	153.0	9.001	0.173	0.188	0.236	0.110	0.408	0.157	0.220

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Experiments

4a9- .27 7 4.8	3 5a5- .37 6.6
4a10- .36 8 6.4	4 5a6- .32 5.7
4a11- .42 9 7.5	5 5a7- .29 5.2
4a12- .25 10 4.45	6 5a8- .28 5.0
4aA- .53 11 9.43	7 5a9- .36 6.4
4aB- .59 12 10.5	8 5a10- .18 3.2
4aC- .56 10.0	9 5a11+12- .21 3.7
4aD- .50 2 8.9	10 5a12- .22 3.9
4aX- .18 3 3.2	11 5a13- .25 4.4
4aY- .18 4 3.2	12 5a14- .21 3.7
4aZ- .22 5 3.9	1 5a15- .17 3.0
4b1- .36 6 6.4	2 5a16- .27 4.8
4b3- .47 7 8.4	3 5a17- .32 5.7
4b4- .30 8 5.3	4 5aA- .25 4.4
4b5- .49 9 8.7	5 5b1- .58 10.3
4b6- .22 10 3.9	6 5b2- .35 6.2
4b7- .20 11 7.1	7 5b3- .37 6.6
4b8- .36 12 8.8	8 5b4- .43 7.6
4b9- .36 1 6.4	9 5b5- .32 5.7
4b10- .38 2 6.7	10 5b6- .32 5.7
4b11- .29 3 5.2	11 5b8- .52 9.2
4b12- .26 4 4.6	12 5b9- .37 6.6
4b13- .25 5 4.4	1 5b10- .17 3.0
4b14- .27 6 4.8	2 5b12- .20 3.6
4b15- .46 7 8.2	3 5b13- .13 2.3
4b16- .42 8 7.5	4 5b14- .30 5.3
4b17- .48 9 8.5	5 5b15- .39 6.4
4b18- .39 10 6.9	6 5b16- .21 3.7
5a1- .40 11 7.1	7 5b17- .20 3.6
5a2- .41 12 7.3	8 5b19- .44 7.8
5a3- .36 1 6.4	9 5b20- .30 5.3
5a4- .38 2 6.8	10 5b22- .16 2.8

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Experiment Title

5b23- .24 11 4.3	7 7a7- .20 3.6
5b24- .31 12 5.5	8 7a8- .26 4.6
5b26- .18 1 3.2	9 7a9+10- .23 4.1
5b27- .25 2 4.4	10 7a10- .20 3.6
5b29- .22 3 3.9	11 7a11- .27 4.8
5b30- .16 4 2.8	12 7a12- .23 4.1
6a1- .32 5 5.7	1 7a14- .35 6.2
6a3- .27 6 4.8	2 7aA- .24 4.3
6a4- .36 7 6.4	3 7aB- .23 4.1
6aA- .32 8 5.7	4 7aC- .16 2.8
6aB- .34 9 6.0	5 7b2- .35 6.2
6aC- .33 10 5.9	6 7b3+5- .39 6.9
6b1- .34 11 6.0	7 7b3- .21 3.7
6b2- .30 12 5.3	8 7b5- .35 6.2
6b3- .25 1 4.4	9 7b6- .34 6.0
6b4- .24 2 4.3	10 7b7- 7aA .26 4.6 7
6b5- .39 3 6.9	11 7b10- .22 3.9
6b6- .26 4 4.6	12 7b11- .39 6.9
6b8- .31 5 5.5	1 7b13- .34 6.0
6b9- .34 6 6.0	2 7b14- .34 6.0
6b10- .43 7 7.6	3 7b15- .25 4.4
6b11- .32 8 5.7	4 7b16- .30 5.3
6b13- .28 9 5.0	5 Controls N°1- .44 7.8
6b14- .52 10 9.2	6 Controls N°2- .55 9.8
6b15- .45 11 8.0	7 extraction
6b16- .31 12 5.5	8 extraction
7a1- .29 1 5.2	
7a2- .24 2 4.3	
7a3- .26 3 4.6	
7a4- .26 4 4.6	
7a5- .24 5 4.3	
7a6- .27 6 4.8	

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## Experiment Title

plate 1  
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	4790	4695	4711	4677	4598	4634	4143	4650	4539	4596	4454	4386
B	4672	4550	4523	4702	4530	4503	4405	4514	4613	4647	4730	4465
C	4996	4710	4876	4862	4768	4693	4725	4790	4566	4631	4695	3041
D	4856	161.5	223.3	3355	994.1	4653	4648	4642	3421	4651	4668	4444
E	4832	4712	103.6	4771	4071	4670	4593	4610	1610	4439	1566	152.1
F	708.2	4645	4641	4823	4577	4545	4638	523.4	326.8	4532	4404	4402
G	389.6	4530	4598	4364	4461	4271	4315	62.14	87.80	15.52	4395	5851
H	4639	3851	4559	4585	4551	280.1	123.1	4429	4459	4453	4361	4320

TRANSF. 4:

plate 2  
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	4729	4525	314.7	4914	1075	4125	4789	4619	4645	4587	4693	4614
B	4614	4771	4775	4793	4766	4766	4709	4565	4711	212.7	4628	4497
C	4606	4743	314.5	4835	110.6	108.0	4651	355.5	4667	4607	390.5	1060
D	4725	4867	4752	4870	4861	4717	3929	152.7	4562	4698	4613	4616
E	4473	277.1	3912	4771	1696	4596	4572	4311	366.4	4429	4501	4466
F	325.9	4678	4684	4732	4658	4704	4650	1340	2678	4486	4590	4438
G	4539	3086	4705	256.5	959.3	494.8	4577	4596	673.9	72.50	4473	4497
H	621.2	4503	4578	162.2	406.1	197.1	4532	438.9	4499	4525	4304	216.6

plate 3  
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	280.6	293.8	8.788	245.8	23.66	0.084						
B	90.59	119.1	8.758	4959	222.9	0.084						
C	149.7	99.73	8.755	47.30	137.9	0.114						
D	44.14	61.30	8.742	124.0	80.24	0.101						
E	382.3	4819	8.758	211.5	14.74	0.185						
F	409.2	4990	8.747	130.5	17.27	0.168						
G	961.6	36.46	8.782	31.88	9.377	0.084						
H	5027	31.89	8.788	154.4	9.024	0.184						

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Experiment Title

plate 1  
Second Reading  
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2836	2682	2873	2792	2805	2762	2469	2803	2718	2699	2637	2555	✓
B	2691	2595	2719	2872	2785	2691	2680	2713	2812	2733	2890	2628	✓
C	2973	2779	3001	2982	2980	2892	2944	2942	2776	2770	2845	1670	✓
D	2857	72.48	107.9	1845	486.0	2801	2888	2834	1930	2785	2885	2669	✓
E	2839	2838	48.92	2896	2357	2865	2791	2790	831.8	2661	808.5	87.91	✓
F	328.4	2713	2826	3024	2797	2807	2929	264.5	166.1	2817	2740	2680	✓
G	174.1	2624	2841	2620	2652	2579	2559	30.00	60.88	53.34	2730	4092	✓
H	2684	2110	2839	2758	2716	134.0	59.37	2696	2700	2705	2628	2594	✓

plate 2  
Second Reading  
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2797	2643	175.8	2976	543.5	2379	2906	2749	2796	2752	2820	2709	✓
B	2682	2837	2883	2916	2885	2900	2842	2690	2868	124.1	2785	2665	✓
C	2691	2833	178.5	2902	76.92	76.45	2800	189.8	2793	2764	207.0	522.1	✓
D	2752	2927	2935	2988	6607	2870	2282	94.69	2723	2861	2807	2777	✓
E	2552	152.4	2268	2978	837.9	2714	2710	2495	187.4	2635	2717	2594	✓
F	174.4	2896	2893	2870	2820	2888	2836	678.0	1426	2705	2809	2657	✓
G	2641	1677	2855	144.3	481.9	257.3	2757	2779	340.6	58.74	2695	2716	✓
H	310.4	2684	2750	101.6	213.7	116.2	2728	231.0	2689	2731	2557	124.3	✓

plate 3  
Second Reading  
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A	152.8	160.8	<del>33.82</del>	140.6	38.10								✓
B	68.14	82.42	<del>33.82</del>	2969	131.9								✓
C	94.68	73.50	<del>33.82</del>	49.31	92.04								✓
D	47.13	56.39	<del>33.82</del>	33.82	66.42								✓
E	197.5	2791	<del>33.82</del>	125.9	34.74								✓
F	211.9	2972	<del>33.82</del>	89.95	36.26								✓
G	459.2	44.20	<del>33.82</del>	43.16	32.10								✓
H	2859	41.82	<del>33.82</del>	100.4	31.91								✓

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## Experiment Title

Calibrated Gas Assay Reading

Note: Figures from the second reading were used. For large figures use the readings from 2x dilution. For small ones, use the reading without dilution. The readings were then divided by the calculated concentration of each sample. Final data

Sample	calibrated reading	Sample	Calibrated Reading
1a1	650	1b15	762
1a2	571	1b16	783
1a3	586	1b17	714
1a4	594	1b18	686
1a5	680	1b19	685
1a6	736	1b20	723
1a7	525	1b20*	647
1aA	534	1b23	891
1aB	544	1b24	730
1aC	500	1b25	676
1b1	1014	2a1	1323
1b2	700	2a2	630

1b3	145-K	2a3	783
1b4	811	2a4	1420 16.2
1b5	929	2a5	23.8
1b6	660	2a6	473
1b7	1160	2a7	99.2
1b8	656	2a8	483
1b9	616	2a9	791
1b10	1023	2a10	623
1b11	987	2a11	433
1b12	547	2a12	977

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Experiment Title

Sample	Calibrated Reading	Sample	Calibrated Reading
2a15	962	3a4	121
2a16	728	3a5	1418
2a17	79.9	3a6	1291
2a18	1016	3a7	1139
2a19	428	3a8	1560
2b1	971	3a9	1121
2b2	979	3aA	752
2b3	664	3b1	10.4
2b4	168	3b2	8.96
2b5	578	3b3	2.82
2b6	155	3b5	958
2b7	19.5	3b6	1320
2b9	164	3b7	716
2b10	1130	3b8	811
2b11	819	3b9	1384
2b12	1634	3b10	1199
2b14	766	3b11	988
2b16	1169	3b12	65.1
2b17	1065	3b14	20.9
2b19	95.2	3b16	490
2bA	76.0	3b17	551
3a1	1760	3b18	1040
3a2	1245	4a1	922
3a3	1675	4a2	1330

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## Experiment Title

Sample	Calibrated Reading	Sample	Calibrated Reading
4a3	745	4b9	841
4a4	361	4b10	846
4a5	57.2	4b11	60.5
4a6	1044	4b12	1261
4a7	207	4b13	25.1
4a8	743	4b14	22.5
4a9	1210	4b15	683.
4a10	859	4b16	47.4
4a11	746	4b17	657
4a12	1223	4b18	801
4aA	598	5a1	55
4aB	516	5a2	145
4aC	536	5a3	860
4aD	637	5a4	861
4aX	1801	5a5	889
4aY	1822	5a6	1048
4aZ	1479	5a7	2541 ✓
4b1	906	5a8	1148
4b3	677	5a9	713
4b4	1015	5a10	47.7
4b5	659	5a11+12	1472
4b6	54.5	5a12	1467
4b7	784	5a13	1275
4b8	833	5a14	1501

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Experiment Title

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Sample	Calibrated Reading	Sample	Calibrated Reading
5a15	170.1	5b26	1650
5a16	57.7	5b27	762
5a17	795	5b29	1464
5aA	1353	5b30	91.6
5b1	165	6a1	168.3
5b2	875	6a3	103.1
5b3	821	6a4	861.
5b4	656	6aA	975
5b5	64.3	6aB	112
5b6	924	6aC	12.3 ✓
5b8	590	6b1	898
5b9	786	6b2	1024
5b10	109	6b3	141.
5b12	1609	6b4	1248
5b13	2515	6b5	797
5b14	1083	6b6	35.3 ✓
5b15	817	6b8	77.8
5b16	1561	6b9	32.8 ✓
5b17	1575	6b10	718
5b19	172	6b11	77.0
5b20	538	6b13	1076
5b22	1932	6b14	594
5b23	1306	6b15	639
5b24	966	6b16	39.3

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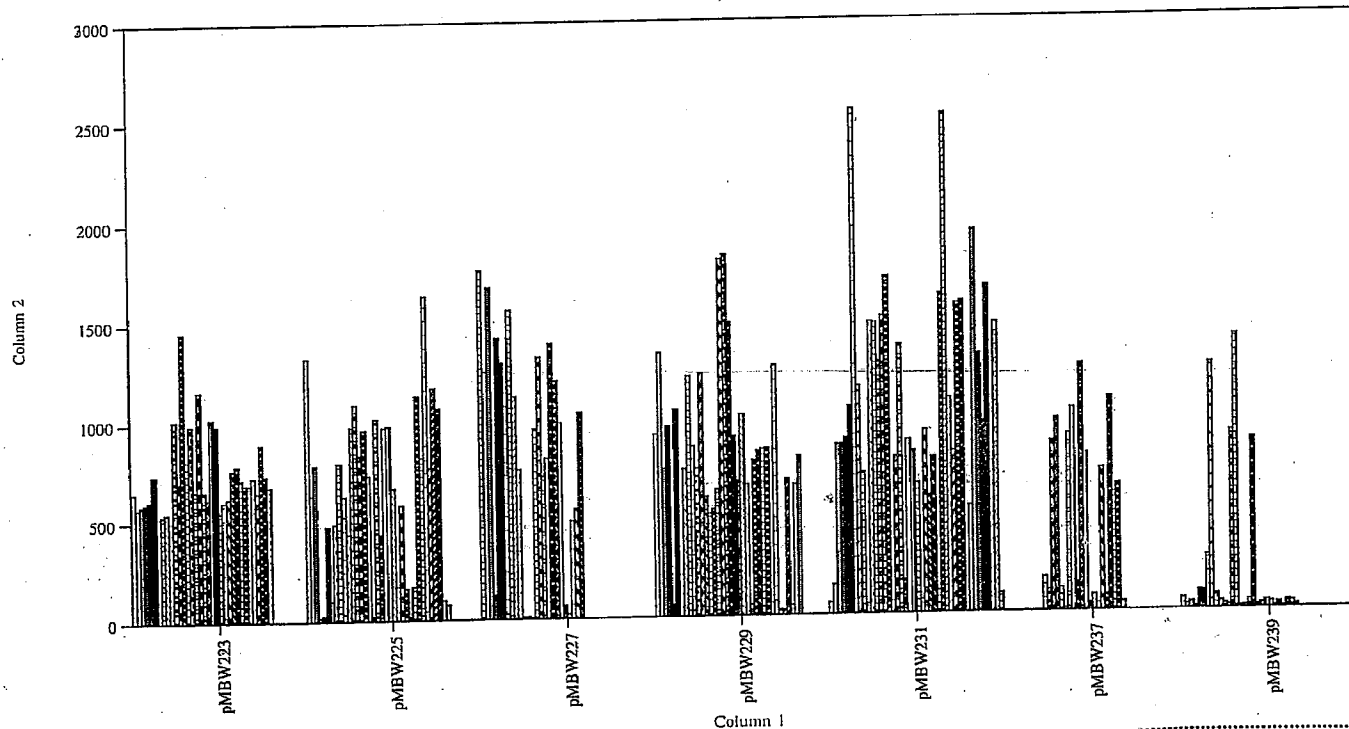
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## Experiment Title

Sample	Calibrated Reading	Sample	Calibrated Reading
7a1	54.0	7b2	39.6
7a2	21.1	7b3+5	860
7a3	32.5	7b3	12.8
7a4	9.60	7b5	20.0
7a5	88.9	7b6	35-2
7a6	85.2	7b7	28-4
7a7	267.1	7b10	8-17
7a8	1243	7b11	22-4
7a9+10	76.6	7b13	3-94
7a10	33.1	7b14	37-2
7a11	20.8	7b15	31-3
7a12	15.0	7b16	15-1
7a14	900.8	control 1	1.89
7aA	1382	control 2	1.76
7aB	8.90	exh. buff.	
7aC	11.4	exh. buff.	

## GUS ASSAY



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Experiment Title Repeat the AUS Assay for supertransformed *V. c* cell  
this time dilute the extract to 1 mg/ml protein, use 1.5  $\mu$ l for  
assay. Measure kinetic value rather than end product

NSF. 2:

NSF. 3:

NSF. 4:

plate No. 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	121.0	45.58	99.28	26.17	92.21	108.8	6.432	90.80	98.24	93.76	109.5	109.5
B	30.35	40.04	105.0	23.05	117.8	118.5	11.27	110.6	19.29	121.0	115.1	77.10
C	106.1	73.12	29.97	50.11	80.34	117.8	10.26	67.52	92.32	27.97	97.45	2.22
D	71.60	0.224	0.321	5.290	0.946	32.44	128.8	31.28	5.023	40.27	36.40	53.24
E	29.15	89.06	0.256	68.23	5.841	93.43	73.19	128.1	1.506	140.6	1.275	0.272
F	0.209	42.05	117.5	118.9	21.44	155.0	190.9	0.773	0.146	38.43	64.16	143.2
G	0.955	68.32	105.6	136.6	140.4	62.38	17.79	0.199	0.197	34.3	128.0	130.3
H	196.2	12.43	140.0	90.13	157.4	0.518	0.231	116.1	43.18	31.06	38.88	115.5

NSF. 2:

NSF. 3:

NSF. 4:

plate No. 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	133.0	98.46	0.502	39.35	1.545	10.36	111.6	14.42	88.06	52.28	30.26	107.5
B	26.75	105.1	135.1	95.04	71.15	31.82	58.08	56.32	44.62	0.454	17.32	73.20
C	20.32	107.4	0.453	87.81	0.299	0.206	12.47	0.216	11.15	19.22	0.290	0.572
D	37.20	53.94	105.5	56.73	60.36	71.12	13.17	0.424	26.98	115.5	149.7	66.75
E	145.8	0.534	9.400	35.33	1.395	152.4	67.42	56.4	12.11	0.508	23.34	76.10
F	0.841	66.12	136.9	67.92	50.32	170.3	108.9	1.241	4.092	169.6	76.88	22.08
G	91.60	7.885	69.76	0.832	1.440	0.721	62.39	109.3	1.154	0.214	21.23	129.3
H	1.181	25.57	87.19	0.407	0.537	0.433	19.23	0.785	38.79	27.84	13.67	0.370

NSF. 2:

NSF. 3:

NSF. 4:

plate No. 3.

	1	2	3	4
A	0.565	0.717	0.472	0.172
B	0.316	0.398	21.49	0.436
C	0.351	0.315	0.256	0.398
D	0.210	0.270	0.296	0.242
E	0.701	16.78	0.452	0.167
F	0.733	67.28	0.422	0.157
G	2.103	0.217	0.196	0.165
H	119.8	0.208	0.294	0.164

NSF. 3:

NSF. 4:

Further 5x dilution of a few

	1	2	3	4	5	6	7
A	82.22	51.06					
B	81.99	41.45					
C	71.57	53.15					
D	52.31	57.41					
E	40.75	68.18					
F	58.59	0.150					
G	55.23	0.140					
H	42.75	0.151					

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Proje  
Exp

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a1		1a3		1a5	1a6		1aA	1aB	1aC	1b1	1b2
B			1b5		1b7	1b8		1b10		1b12	1b13	1b14
C	1b15	1b16			1b19	1b20	1b20*	1b23	1b24		2a1	
D	2a3						2a9					
E		2a16		2a18		2b1	2b2	2b3		2b5		2a14
F			2b11	2b12		2b16	2b17					
G		3a5	3a6	3a7	3a8	3a9					3a2	3a3
H	3b7			3b9	3b10			3b16			3b5	3b6
												4a2

ISF. 3:  
ISF. 4:

plate No. 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	4a3	4a4					4a9		4a11			4aB
B		4aD	4aX	4aY	4aZ							4b8
C		4b10		4b12								
D			5a5			5a8				5a12	5a13	5a14
E	5a15					5b2	5b3				5b8	5b9
F		5b12	5b13	5b14		5b16	5b17			5b22	5b23	
G	5b26		5b29				6a4	6aA				6b2
H			6b5									

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Run Time: 00:29:58

Time: 00:00:00-00:29:58

ISF. 3:  
ISF. 4:

plate No. 3

ISF. 3:  
ISF. 4:

Further rx dilution of a few sample

	1	2	3	4
A				
B				
C				
D				
E				
F		7aA		
G				
H	7a8			

	1	2	3	4	5	6	7	8	9
A	1b1	3b7							
B	1b5	3b11							
C	1b7	5b13							
D	1b20*	5b16							
E	2b5	5b22							
F	2b17								
G	3a3								
H	3b6								

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FI: 0.000 - 1000.000

Run Time:  
Time:

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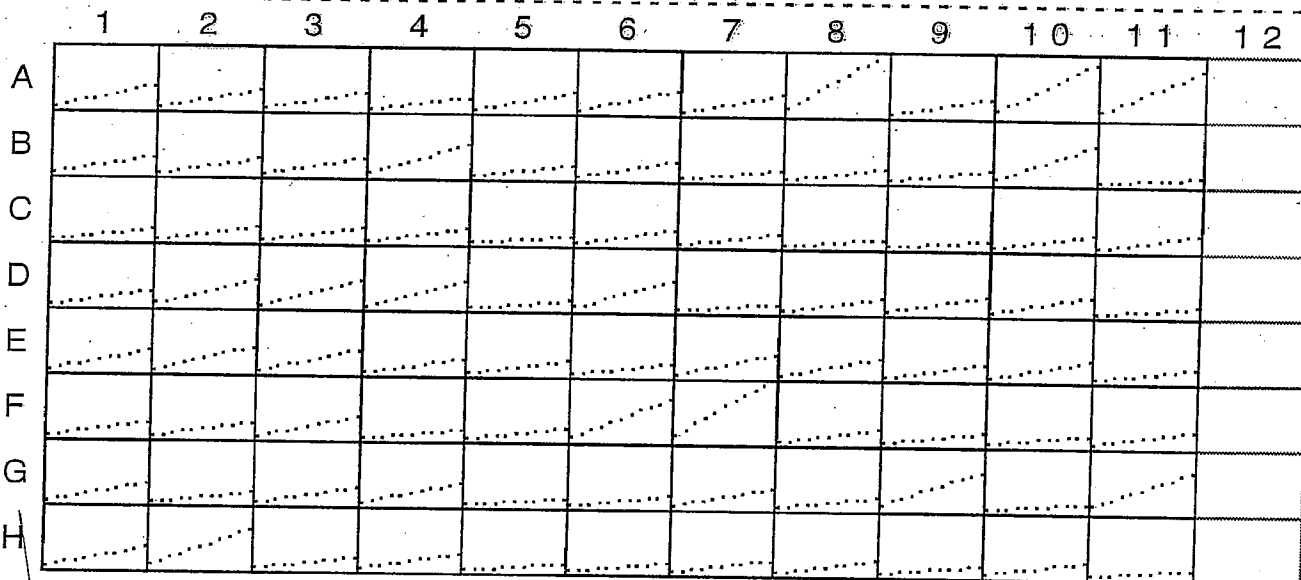
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Experiment Title

Repeat alls Assay for those with too high readings (with red numbering) of yesterday's experiments.

Dilute 5x of the solution used for yesterday's Assay, Use 1.5  $\mu$ l for each assay.



Read Interval: 00:02:00

Run Time: 00:20:00

FI: 0.000 - 1000.000

Time: 00:00:00-00:20:00

	1	2	3	4	5	6	7	8	9	10	11	12
A	16.89	13.90	13.04	9.804	14.04	16.44	13.71	44.92	13.02	39.14	33.97	
B	13.21	11.84	13.26	24.49	9.566	13.41	7.184	8.893	8.579	27.22	4.855	
C	7.982	10.47	9.789	10.41	5.600	11.05	8.823	5.965	5.442	9.739	20.62	
D	11.24	19.73	20.59	21.02	6.334	22.91	5.853	9.915	12.79	13.22	6.288	
E	15.40	18.74	16.94	11.34	9.949	9.317	16.84	14.69	10.74	13.22	8.365	
F	11.35	11.34	16.02	7.297	9.181	31.19	47.35	9.792	8.264	7.410	10.49	
G	14.42	8.307	12.05	16.12	5.499	7.863	14.70	8.251	27.26	5.649	28.11	
H	15.65	28.87	8.750	12.60	5.594	7.089	9.247	9.937	6.743	10.10	5.212	

These figures are generally lower than expected (or yesterday's results)

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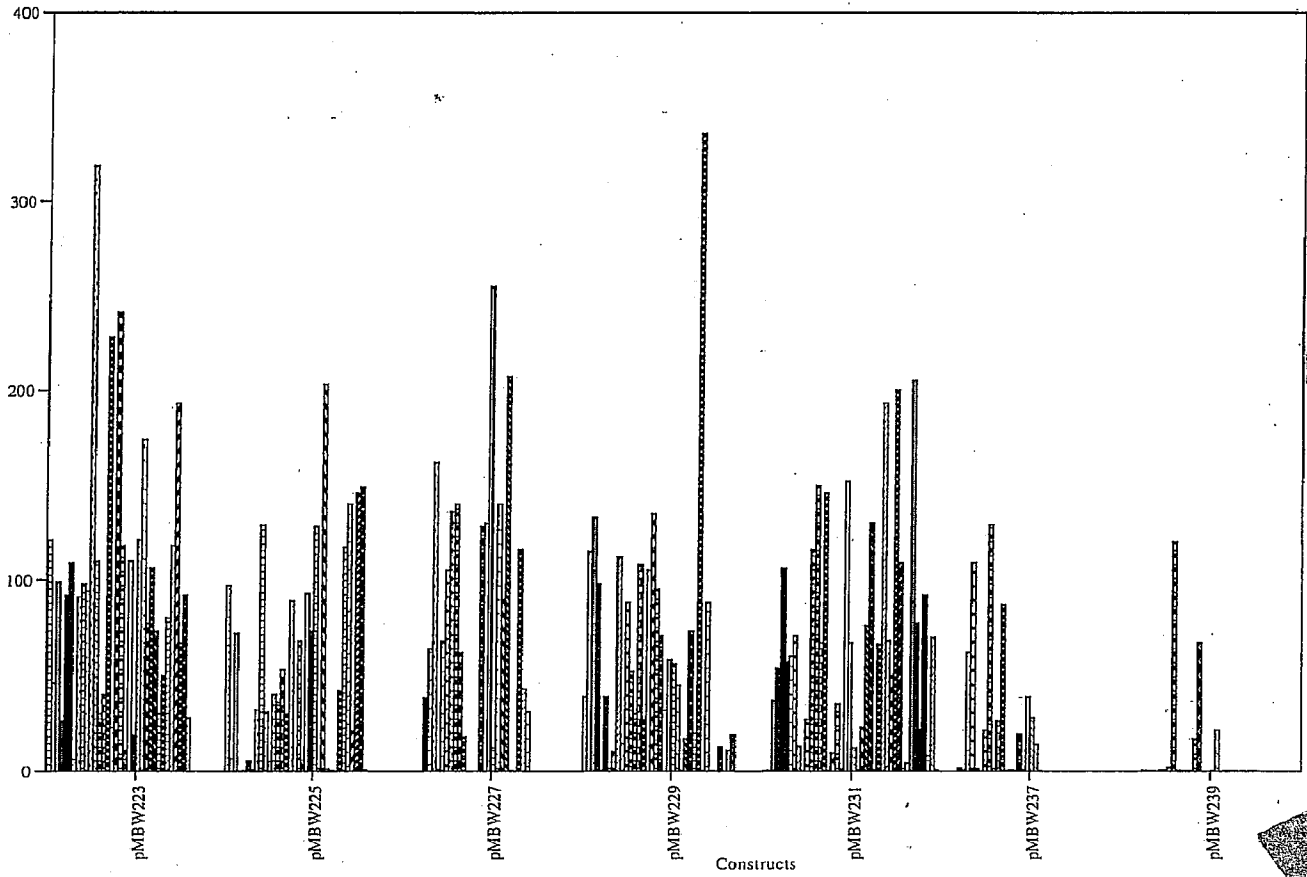
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named after the place of its invention in Eugene, Oregon. The model is a set of three partial differential equations that describe the reaction-diffusion process. Showalter and colleagues added a term to account for the photosensitive generation of bromide ions, and predict wave propagation patterns remarkably similar to those observed in the experiment. Before this experiment, STSR had been studied only theoretically or by numerical or electronic simulation in one-dimensional sets of coupled<sup>6,7</sup> and uncoupled<sup>8</sup> elements, and in two-dimensional arrays of threshold elements<sup>9</sup>. But those 2D simulations, in spite of their simplicity, mimic all the features of the present experiment.

The implications of the present experiment extend far beyond chemical dynamics. Spiral waves, spontaneously generated by noise, have also been simulated with the Oregonator (Fig. 1b). They are strikingly similar to recent observations of noise-initiated and sustained long-range coherent waves of calcium ions in cultured brain tissue<sup>10</sup> (Fig. 1c) indicating a similar under-

lying dynamical process. The possibility that calcium waves transmit or coordinate information over centimetre distances in glial cell networks (that is, in the brain) has already been suggested, but the role of noise remained obscure. Now that noise-sustained spiral waves have been observed in a well characterized chemical system, we can speculate that spatiotemporal noise may be an important feature of the brain's working. □

Frank Moss is at the Center for Neurodynamics, University of Missouri at St Louis, St Louis, Missouri 63121, USA.

e-mail: mossf@umslvma.umsl.edu

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## Functional genomics

# Double-stranded RNA poses puzzle

Richard W. Wagner and Lin Sun

The human genome is predicted to contain between 50,000 and 100,000 genes<sup>1</sup>. To work out what these genes do, an array of techniques is needed to evaluate the protein-protein interactions and biochemical pathways of any gene product. The nematode worm *Caenorhabditis elegans* is an excellent system for such studies because of its well-understood genetics and development, evolutionary conservation to human genes, small genome size and relatively short life cycle. The 100-megabase-pair genome will be completely sequenced this year, and a total of 17,000 genes have been predicted, many with human counterparts. Approaches used to manipulate gene expression in *C. elegans* include transposon-mediated deletion<sup>2</sup>, antisense inhibition<sup>3</sup> and direct isolation of deletions after mutagenesis<sup>4,5</sup>. Although these methods have proved useful, limitations still exist.

On page 806 of this issue, Fire and colleagues<sup>6</sup> describe a remarkable and surprising technique for inhibiting gene function in *C. elegans*. They turned off a specific gene in progeny worms by microinjecting double-stranded RNA (dsRNA) complementary to the coding region of the gene into the gonads of adult animals. Using a well-characterized gene, *unc-22*, which encodes a non-essential myofibril protein, they showed that injection of dsRNA produced a phenotype

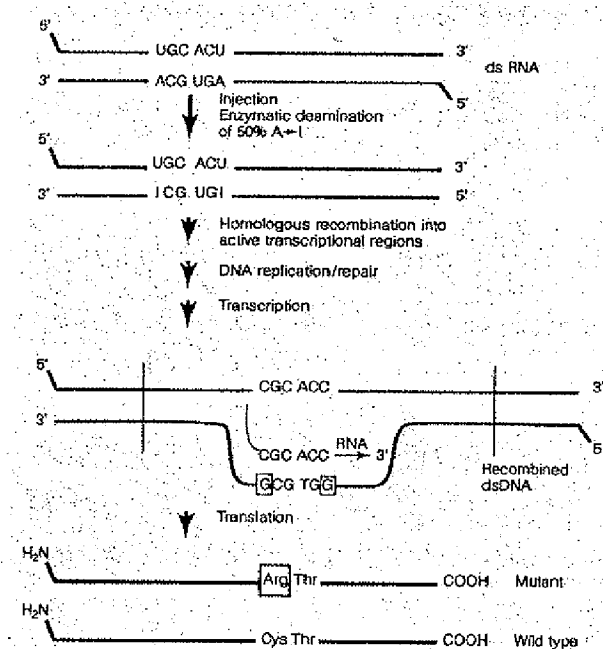
characteristic of *unc-22* inhibition—twitching.

In a series of well-controlled studies, the authors also found that injection of dsRNA targeted to a reporter gene for green fluorescent protein resulted in a dramatic — and

specific — decrease in protein production. Furthermore, when they injected dsRNA targeted to another gene, *mex-3*, the result was a loss of *mex-3* RNA in early-stage embryos. In other words, at the levels of phenotype, RNA and protein, the interference with gene expression was specific and reproducible.

Perhaps most astounding is the phenomenon that the dsRNA causes gene inhibition. Previously<sup>3</sup>, Fire and co-workers had been puzzled by the fact that antisense RNA alone — which is often used to inactivate sense messenger RNA — was only marginally effective. Furthermore, results using the antisense RNA were mimicked by injection of sense RNA, a control in their studies. They later found out that these data could be largely explained by an artefact of the transcription process that was used to generate the antisense and sense RNAs; namely, dsRNA fragments.

Additional experiments by Fire *et al.*, designed to shed light on the possible mechanism of the dsRNA-mediated inhibition, painted an even more mystifying picture. For example, even when only a few copies of the dsRNAs are present in each cell, they are active against highly abundant RNAs. This indicates that the interference occurs either by a catalytic mechanism or at the chromosomal level — and not by a conventional antisense mechanism. The authors also found that only dsRNAs that are complementary to coding regions of the gene are active, and not, for example, those targeted to introns or promoter regions. This argues against a generalized mechanism involving chromosomal inactivation, such as chromosomal deletion. Moreover, dsRNA interference seems to cross cellular boundaries with



**Figure 1** Possible mechanism for inhibition of gene expression in *C. elegans* by double-stranded RNA. Fire *et al.*<sup>6</sup> have convincingly shown that, at the phenotype, RNA and protein levels, dsRNA-mediated interference with gene expression is specific and reproducible. Perhaps, on injection into worms, dsRNA is modified by dsRNA adenosine deaminase. Transfer of this information back into the chromosome may occur by a recombination event. After replication and mismatch repair, transcription and translation result in mutant proteins that have impaired function.

ease. Gene inhibition was observed in progeny when dsRNA was injected into the body cavity of the adult (gonadal injections had been thought to be necessary), and in somatic tissues of young adults after injection into their body cavity.

What kind of mechanism have Fire and colleagues uncovered? This is not the first puzzle posed by dsRNA. Almost ten years ago, Bass and Weintraub<sup>7</sup> and Wagner *et al.*<sup>8</sup> discovered an enzyme that binds dsRNA and deaminates adenosines in the duplex to inosines. After a feverish hunt for the cellular function of the dsRNA adenosine deaminase, it was found to be involved in the post-transcriptional editing of messages. Inosines are read by the cellular machinery as guanosine, so the enzyme could alter the genetic make-up of mRNA (reviewed in refs 9, 10).

Could this dsRNA adenosine deaminase be involved in a complicated pathway that results in gene inhibition in *C. elegans*? Quite possibly. The enzymatic activity has been found in *C. elegans*, and would probably treat the injected dsRNA as a substrate. A specialized homologous recombination system would be needed, which would use the modified dsRNA to transfer the genetic alterations into the chromosome (Fig. 1).

This model fits some of the data: modification of adenosines to inosines alters the genetic make-up of the injected dsRNA; transfer of this information into the genome by recombination would affect coding (but not intronic) regions; and mutations introduced by the inosine substitutions would affect the ability to detect mRNA and, at least partially, the function of the protein. These mutations could account for the surprising result that only a few copies of dsRNA are required per cell, because they would have an effect at the level of the chromosome. Of course, such a model is a stretch of the imagination and is not supported by all of the data. For example, attempts to use homologous recombination with dsDNA in *C. elegans* have largely failed<sup>9</sup>.

Fire and colleagues<sup>6</sup> have uncovered a complex and intriguing mode of regulation in *C. elegans*. Does dsRNA perform a biological function in *C. elegans* (and is this function titrated out by the microinjected dsRNA)? Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene? A similar mode of action would not be suspected to occur in mammals, because injection of dsRNA is often used as a control for antisense experiments, albeit at the individual cell (and not organism) level. Nevertheless, perhaps specific 'knockouts' can be generated this way, for organisms in which genetic material cannot be delivered by microinjection. Whatever the mechanism might be, dsRNA-

mediated inhibition of gene expression will provide a useful alternative for working out gene function in *C. elegans* and, maybe, in other animals and plants.

Richard W. Wagner and Lin Sun are at Phyllos Inc., 300 Putnam Avenue, Cambridge, Massachusetts 02139, USA.

e-mail: rwagner@phyllos.com

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## Liquid crystals

# New designs in cholesteric colour

Peter Palffy-Muhoray

Since their discovery in 1888, cholesteric liquid crystals have been subject to considerable attention, resulting in applications in ink and paint technologies, flat-panel displays and thermal imaging. Writing in *Advanced Materials*<sup>1</sup>, Tamaoki and co-workers describe a new technique for rewritable full-colour image recording on thin cholesteric films. The low-molecular-weight compound they have developed for this purpose is a cholesteric glass, which is stable at room temperature and which could have applications in optics as well as infor-

mation display and storage.

The optical properties of cholesterics have made them useful in display<sup>2,3</sup> and laser technologies<sup>4</sup> as well as in the visual arts<sup>5</sup>. In reflected light, cholesterics show intense iridescent colours with a metallic sheen, as seen on scarab beetles. In these materials, rod-like molecules are orientated, on the average, parallel to one another in a given plane, so that the direction of orientation varies linearly with position in the direction normal to the plane. This results in a spatially periodic twisted helical structure as shown in

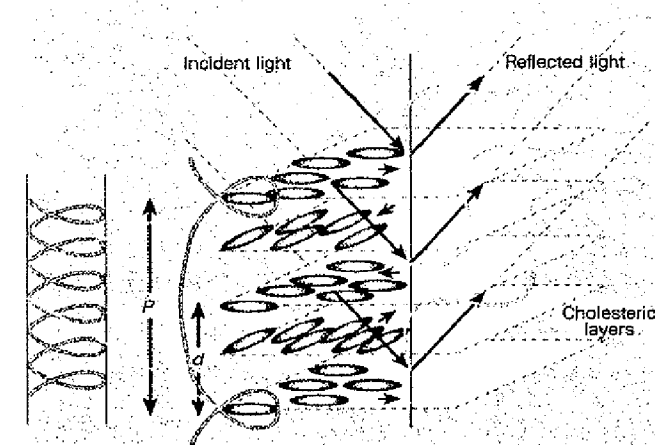


Figure 1 Sketch of cholesteric structure, showing the dependence of molecular orientation on position. The tip of a vector indicating local molecular orientation traces out a helix. Reflected light waves satisfying the Bragg condition emerge in-phase and add constructively. In the work discussed here, Tamaoki *et al.*<sup>1</sup> have developed a cholesteric glass that is rewritable and stable at room temperature (see Fig. 3).



Figure 2 Transmission electron micrograph of freeze-fractured helical cholesteric. The pitch is 240 nm. (From ref. 12.)

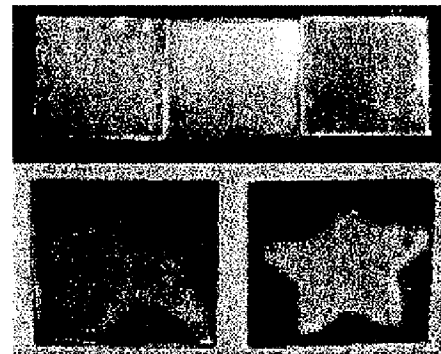


Figure 3 Photographs of thermally addressed and quenched cholesteric solid films. (From ref. 1.)

# Exhibit 25



## The Nobel Prize in Physiology or Medicine 2006



**Nobelförsamlingen**

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## Press Release

2 October 2006

The Nobel Assembly at Karolinska Institutet has today decided to award  
The Nobel Prize in Physiology or Medicine for 2006 jointly to

**Andrew Z. Fire and Craig C. Mello**

for their discovery of **"RNA interference – gene silencing by double-stranded RNA"**

## Summary

This year's Nobel Laureates have discovered a fundamental mechanism for controlling the flow of genetic information. Our genome operates by sending instructions for the manufacture of proteins from DNA in the nucleus of the cell to the protein synthesizing machinery in the cytoplasm. These instructions are conveyed by messenger RNA (mRNA). In 1998, the American scientists Andrew Fire and Craig Mello published their discovery of a mechanism that can degrade mRNA from a specific gene. This mechanism, RNA interference, is activated when RNA molecules occur as double-stranded pairs in the cell. Double-stranded RNA activates biochemical machinery which degrades those mRNA molecules that carry a genetic code identical to that of the double-stranded RNA. When such mRNA molecules disappear, the corresponding gene is silenced and no protein of the encoded type is made.

RNA interference occurs in plants, animals, and humans. It is of great importance for the regulation of gene expression, participates in defense against viral infections, and keeps jumping genes under control. RNA interference is already being widely used in basic science as a method to study the function of genes and it may lead to novel therapies in the future.

## The flow of information in the cell: from DNA via mRNA to protein

The genetic code in DNA determines how proteins are built. The instructions contained in the DNA are copied to mRNA and subsequently used to synthesize proteins (Fig 1). This flow of genetic information from DNA via mRNA to protein has been termed the central dogma of molecular biology by the British Nobel Laureate Francis Crick. Proteins are involved in all processes of life, for instance as enzymes digesting our food, receptors receiving signals in the brain, and as antibodies defending us against bacteria.

Our genome consists of approximately 30,000 genes. However, only a fraction of them are

used in each cell. Which genes are expressed (*i.e.* govern the synthesis of new proteins) is controlled by the machinery that copies DNA to mRNA in a process called transcription. It, in turn, can be modulated by various factors. The fundamental principles for the regulation of gene expression were identified more than 40 years ago by the French Nobel Laureates François Jacob and Jacques Monod. Today, we know that similar principles operate throughout evolution, from bacteria to humans. They also form the basis for gene technology, in which a DNA sequence is introduced into a cell to produce new protein.

Around 1990, molecular biologists obtained a number of unexpected results that were difficult to explain. The most striking effects were observed by plant biologists who were trying to increase the colour intensity of the petals in petunias by introducing a gene inducing the formation of red pigment in the flowers. But instead of intensifying the colour, this treatment led to a complete loss of colour and the petals turned white! The mechanism causing these effects remained enigmatic until Fire and Mello made the discovery for which they receive this year's Nobel Prize.

### **The discovery of RNA interference**

Andrew Fire and Craig Mello were investigating how gene expression is regulated in the nematode worm *Caenorhabditis elegans* (Fig. 2). Injecting mRNA molecules encoding a muscle protein led to no changes in the behaviour of the worms. The genetic code in mRNA is described as being the 'sense' sequence, and injecting 'antisense' RNA, which can pair with the mRNA, also had no effect. But when Fire and Mello injected sense and antisense RNA together, they observed that the worms displayed peculiar, twitching movements. Similar movements were seen in worms that completely lacked a functioning gene for the muscle protein. What had happened?

When sense and antisense RNA molecules meet, they bind to each other and form double-stranded RNA. Could it be that such a double-stranded RNA molecule silences the gene carrying the same code as this particular RNA? Fire and Mello tested this hypothesis by injecting double-stranded RNA molecules containing the genetic codes for several other worm proteins. In every experiment, injection of double-stranded RNA carrying a genetic code led to silencing of the gene containing that particular code. The protein encoded by that gene was no longer formed.

After a series of simple but elegant experiments, Fire and Mello deduced that double-stranded RNA can silence genes, that this RNA interference is specific for the gene whose code matches that of the injected RNA molecule, and that RNA interference can spread between cells and even be inherited. It was enough to inject tiny amounts of double-stranded RNA to achieve an effect, and Fire and Mello therefore proposed that RNA interference (now commonly abbreviated to RNAi) is a catalytic process.

Fire and Mello published their findings in the journal *Nature* on February 19, 1998. Their discovery clarified many confusing and contradictory experimental observations and revealed a natural mechanism for controlling the flow of genetic information. This heralded the start of a new research field.

### **The RNA interference machinery is unraveled**

The components of the RNAi machinery were identified during the following years (Fig 3). Double-stranded RNA binds to a protein complex, Dicer, which cleaves it into fragments. Another protein complex, RISC, binds these fragments. One of the RNA strands is eliminated but the other remains bound to the RISC complex and serves as a probe to detect mRNA molecules. When an mRNA molecule can pair with the RNA fragment on RISC, it is bound to the RISC complex, cleaved and degraded. The gene served by this particular mRNA has been

silenced.

### **RNA interference – a defense against viruses and jumping genes**

RNA interference is important in the defense against viruses, particularly in lower organisms. Many viruses have a genetic code that contains double-stranded RNA. When such a virus infects a cell, it injects its RNA molecule, which immediately binds to Dicer (Fig 4A). The RISC complex is activated, viral RNA is degraded, and the cell survives the infection. In addition to this defense, higher organisms such as man have developed an efficient immune defense involving antibodies, killer cells, and interferons.

Jumping genes, also known as transposons, are DNA sequences that can move around in the genome. They are present in all organisms and can cause damage if they end up in the wrong place. Many transposons operate by copying their DNA to RNA, which is then reverse-transcribed back to DNA and inserted at another site in the genome. Part of this RNA molecule is often double-stranded and can be targeted by RNA interference. In this way, RNA interference protects the genome against transposons.

### **RNA interference regulates gene expression**

RNA interference is used to regulate gene expression in the cells of humans as well as worms (Fig 4B). Hundreds of genes in our genome encode small RNA molecules called microRNAs. They contain pieces of the code of other genes. Such a microRNA molecule can form a double-stranded structure and activate the RNA interference machinery to block protein synthesis. The expression of that particular gene is silenced. We now understand that genetic regulation by microRNAs plays an important role in the development of the organism and the control of cellular functions.

### **New opportunities in biomedical research, gene technology and health care**

RNA interference opens up exciting possibilities for use in gene technology. Double-stranded RNA molecules have been designed to activate the silencing of specific genes in humans, animals or plants (Fig 4C). Such silencing RNA molecules are introduced into the cell and activate the RNA interference machinery to break down mRNA with an identical code.

This method has already become an important research tool in biology and biomedicine. In the future, it is hoped that it will be used in many disciplines including clinical medicine and agriculture. Several recent publications show successful gene silencing in human cells and experimental animals. For instance, a gene causing high blood cholesterol levels was recently shown to be silenced by treating animals with silencing RNA. Plans are underway to develop silencing RNA as a treatment for virus infections, cardiovascular diseases, cancer, endocrine disorders and several other conditions.

#### **Reference:**

Fire A., Xu S.Q., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998; 391:806-811.

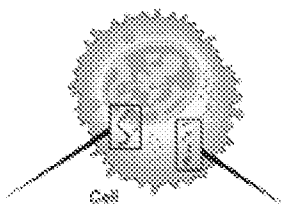
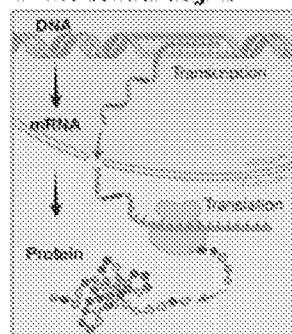
**Andrew Z. Fire**, born 1959, US citizen, PhD in Biology 1983, Massachusetts Institute of Technology, Cambridge, MA, USA. Professor of Pathology and Genetics, Stanford University School of Medicine, Stanford, CA, USA.

**Craig C. Mello**, born 1960, US citizen, PhD in Biology 1990, Harvard University, Boston, MA, USA. Professor of Molecular Medicine and Howard Hughes Medical Institute Investigator, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, USA.

## RNA interference

— gene silencing by double-stranded RNA

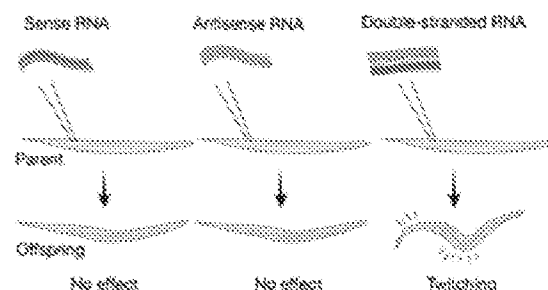
### 1. The central dogma



Our genome operates by sending information from double-stranded DNA in the nucleus, via single-stranded mRNA, to guide the synthesis of proteins in the cytoplasm.

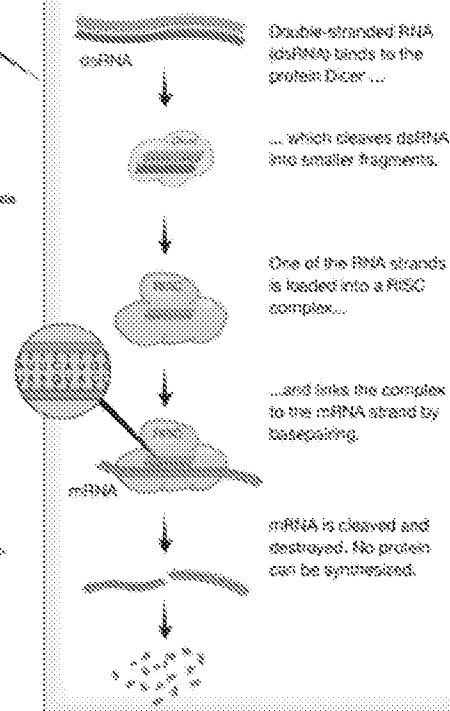
### 2. The experiment

RNA carrying the code for a muscle protein is injected into the worm *C. elegans*. Single-stranded RNA has no effect. But when double-stranded RNA is injected, the worm starts twitching in a similar way to worms carrying a defective gene for the muscle protein.



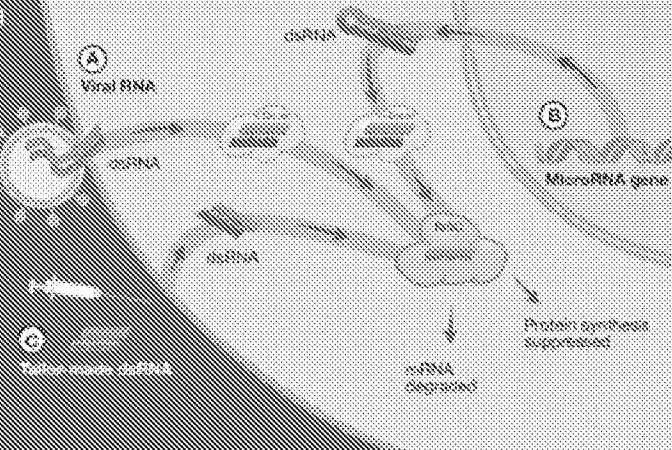
### 3. The RNAi mechanism

RNA interference (RNAi) is an important biological mechanism in the regulation of gene expression.



### 4. Several processes in the cell use RNAi

- When an RNA virus infects the cell, it inserts its genome consisting of double-stranded RNA. RNA interference recognizes the viral RNA, preventing the formation of new viruses.
- Synthesis of many proteins is controlled by genes encoding microRNA. After processing, microRNA prevents the translation of mRNA to protein.
- In the research laboratory, dsRNA molecules are often made to activate the RISC complex to degrade mRNA for a specific gene.



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and 10 Na<sub>2</sub>HPO<sub>4</sub>. FV solution also contained 0.2 NaF and 0.1 Na<sub>2</sub>VO<sub>4</sub>. Rarely, irreversible current rundown still occurred with FVPP. The total Na<sup>+</sup> concentration of all cytoplasmic solutions was adjusted to 30 mM with NaOH, and pH was adjusted to 7.0 with N-methylglucamine (NMG) or HCl. PIP<sub>2</sub> liposomes (20–200 nm) were prepared by sonicating 1 mM PIP<sub>2</sub> (Boehringer Mannheim) in distilled water. Reconstituted monoclonal PIP<sub>2</sub> antibody (Perspective Biosystems, Framingham, MA) was diluted 40-fold into experimental solution. Current-voltage relations of all currents reversed at E<sub>K</sub> and showed characteristic rectification, mostly owing to the presence of Na<sup>+</sup> in FVPP and possibly also residual polyamines. Current records presented (measured at 30 °C, –30 mV holding potential) are digitized strip-chart recordings. Purified bovine brain Gβγ<sup>29</sup> was diluted just before application such that the final detergent (CHAPS) concentration was 5 μM. Detergent-containing solution was washed away thoroughly before application of PIP<sub>2</sub>, because application of phospholipid vesicles in the presence of detergent usually reversed the effects of Gβγ; presumably, Gβγ can be extracted from membranes by detergent plus phospholipids.

**Molecular biology.** R188Q mutation was constructed by insertion of the mutant oligonucleotides between the BsmI and BglII sites of pSPORT-ROMK1 (ref. 11). A polymerase chain reaction (PCR) fragment (amino acids 180–391) from pSPORT-ROMK1 R188Q mutant was subcloned into pGEX-2T vector (Pharmacia) for expression of R188Q mutant protein of GST-RKC. The construction, expression and purification of GST-IRK1 (amino acids 182–428 of IRK1), GST-GKC (180–462 of GIRK1), GST-IKN (1–86 of IRK1) have been described<sup>21,22</sup>.

**In vitro PIP<sub>2</sub> binding assay.** <sup>3</sup>H-PIP<sub>2</sub> in chloroform-methanol (1:1) (American Radiolabeled Chemicals; 0.4 μCi nM<sup>-1</sup> specific activity) was dried under N<sub>2</sub> and sonicated in 100 μl phosphate buffered saline (PBS) to form pure <sup>3</sup>H-PIP<sub>2</sub> liposomes. Purified GST fusion protein (100 nM) was incubated with <sup>3</sup>H-PIP<sub>2</sub> (0.2–1 μM) and precipitated by glutathione 4B-Sepharose beads. After 1 wash with PBS, the precipitates were dissolved in SDS gel loading buffer and counted in a beta-scintillation counter using a window for <sup>3</sup>H. The bound <sup>3</sup>H radioactivity was typically in the range ~2–8% of the total added. For co-immunoprecipitation, 25% PIP<sub>2</sub> or PIP in 75% phosphatidylcholine (PC) background (30 μg PIP<sub>2</sub> or PIP (Boehringer Mannheim) and 90 μg phosphatidylcholine (Sigma)), both in chloroform, were dried down together and sonicated in 300 μl PBS to form mixed liposome. GST fusion proteins were first incubated with 25% PIP<sub>2</sub> or PIP liposome (100 μM) and PIP<sub>2</sub> antibodies (1:100 dilution) for 2 h and with protein A-Sepharose for a further 30 min. After one wash with PBS, the immunoprecipitates were separated by 10% SDS-PAGE, probed with specific antibodies<sup>21,22</sup>, and visualized by ECL (Amersham). Each experiment was performed at least twice with similar results. The relative amount of immunoreactivity in each lane was quantified by serial dilutions of sample<sup>21</sup>.

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Correspondence and requests for materials should be addressed to C.L.H. (e-mail: chuanl@mednet.swmed.edu).

## Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire\*, SiQun Xu\*, Mary K. Montgomery\*, Steven A. Kostas††, Samuel E. Driver‡ & Craig C. Mello†

\* Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA  
† Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA  
‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene<sup>1,2</sup>. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression<sup>3,4</sup>. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stoichiometric interference with endogenous

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mRNA and suggesting that there could be a catalytic or amplification component in the interference process.

Despite the usefulness of RNA interference in *C. elegans*, two features of the process have been difficult to explain. First, sense and antisense RNA preparations are each sufficient to cause interference<sup>3,4</sup>. Second, interference effects can persist well into the next generation, even though many endogenous RNA transcripts are rapidly degraded in the early embryo<sup>5</sup>. These results indicate a fundamental difference in behaviour between native RNAs (for example, mRNAs) and the molecules responsible for interference. We sought to test the possibility that this contrast reflects an underlying difference in RNA structure. RNA populations to be injected are

generally prepared using bacteriophage RNA polymerases<sup>6</sup>. These polymerases, although highly specific, produce some random or ectopic transcripts. DNA transgene arrays also produce a fraction of aberrant RNA products<sup>7</sup>. From these facts, we surmised that the interfering RNA populations might include some molecules with double-stranded character. To test whether double-stranded character might contribute to interference, we further purified single-stranded RNAs and compared interference activities of individual strands with the activity of a deliberately prepared double-stranded hybrid.

The *unc-22* gene was chosen for initial comparisons of activity. *unc22* encodes an abundant but nonessential myofilament protein<sup>7-9</sup>. Several thousand copies of *unc-22* mRNA are present in each

Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F <sub>1</sub> phenotype
<i>unc-22</i>				<i>unc-22</i> -null mutants: strong twitchers <sup>18</sup>
<i>unc22A</i> *	Exon 21-22	742	Sense Antisense Sense + antisense	Wild type Wild type Strong twitchers (100%)
<i>unc22B</i>	Exon 27	1,033	Sense Antisense Sense + antisense	Wild type Wild type Strong twitchers (100%)
<i>unc22C</i>	Exon 21-22†	785	Sense + antisense	Strong twitchers (100%)
<i>fem-1</i>				<i>fem-1</i> -null mutants: femal (no sperm) <sup>19</sup>
<i>fem1A</i>	Exon 10‡	531	Sense Antisense Sense + antisense	Hermaphrodite (98%) Hermaphrodite (>98%) Female (72%)
<i>fem1B</i>	Intron 8	556	Sense + antisense	Hermaphrodite (>98%)
<i>unc-54</i>				<i>unc-54</i> -null mutants: paralysed <sup>20</sup>
<i>unc54A</i>	Exon 6	576	Sense Antisense Sense + antisense	Wild type (100%) Wild type (100%) Paralysed (100%)§
<i>unc54B</i>	Exon 6	651	Sense Antisense Sense + antisense	Wild type (100%) Wild type (100%) Paralysed (100%)§
<i>unc54C</i>	Exon 1-5	1,015	Sense + antisense	Arrested embryos and larvae (100%)
<i>unc54D</i>	Promoter	567	Sense + antisense	Wild type (100%)
<i>unc54E</i>	Intron 1	369	Sense + antisense	Wild type (100%)
<i>unc54F</i>	Intron 3	386	Sense + antisense	Wild type (100%)
<i>hlh-1</i>				<i>hlh-1</i> -null mutants: lumpy-dumpy larvae <sup>16</sup>
<i>hlh1A</i>	Exons 1-6	1,033	Sense Antisense Sense + antisense	Wild type (<2% lpy-dpy) Wild type (<2% lpy-dpy) Lpy-dpy larvae (>90%)
<i>hlh1B</i>	Exons 1-2	438	Sense + antisense	Lpy-dpy larvae (>80%)
<i>hlh1C</i>	Exons 4-6	299	Sense + antisense	Lpy-dpy larvae (>80%)
<i>hlh1D</i>	Intron 1	697	Sense + antisense	Wild type (<2% lpy-dpy)
<i>myo-3</i> -driven GFP transgenes†				Makes nuclear GFP in body muscle
<i>myo-3::NLS::gfp::lacZ</i>				Nuclear GFP-LacZ pattern of parent strain
<i>gfpG</i>	Exons 2-5	730	Sense Antisense Sense + antisense	Nuclear GFP-LacZ pattern of parent strain Nuclear GFP-LacZ absent in 98% of cells
<i>lacZL</i>	Exon 12-14	830	Sense + antisense	Nuclear GFP-LacZ absent in >95% of cells
<i>myo-3::MtLS::gfp</i>				Makes mitochondrial GFP in body muscle
<i>gfpG</i>	Exons 2-5	730	Sense Antisense Sense + antisense	Mitochondrial-GFP pattern of parent strain Mitochondrial-GFP pattern of parent strain Mitochondrial-GFP absent in 98% of cells
<i>lacZL</i>	Exon 12-14	830	Sense + antisense	Mitochondrial-GFP pattern of parent strain

Each RNA was injected into 6-10 adult hermaphrodites ( $0.5 \times 10^6 - 1 \times 10^6$  molecules into each gonad arm). After 4-6 h (to clear preferentially eggs from the uterus), injected animals were transferred and eggs collected for 20-22 h. Progeny phenotypes were scored upon hatching and subsequently at 12-24-h intervals.

\* to obtain a semiquantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations (see figure in Supplementary information for details). At the highest dose tested ( $3.6 \times 10^6$  molecules per gonad), the individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny, respectively). Comparable doses of double-stranded *unc22A* RNA produced visible twitching in all progeny, whereas a 120-fold lower dose of double-stranded *unc22A* RNA produced visible twitching in 30% of progeny. † *unc22C* also carries the 43-nucleotide intron between exons 21 and 22. ‡ *fem1A* carries a portion (131 nucleotides) of intron 10. § Animals in the first affected broods (laid 4-24 h after injection) showed movement defects indistinguishable from those of *unc-54*-null mutants. A variable fraction of these animals (25%-75%) failed to lay eggs (another phenotype of *unc-54*-null mutants), whereas the remainder of the paralysed animals did lay eggs. This may indicate incomplete interference with *unc-54* activity in vulval muscles. Animals from later broods frequently show a distinct partial loss-of-function phenotype, with contractility in a subset of body-wall muscles. || Phenotypes produced by RNA-mediated interference with *hlh-1* included arrested embryos and partially elongated L1 larvae (the *hlh-1*-null phenotype). These phenotypes were seen in virtually all progeny after injection of double-stranded *hlh1A* and in about half of the affected animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*. A set of less severe defects was seen in the remainder of the animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*. The less severe phenotypes are characteristic of partial loss of function of *hlh-1* (B. Harfe and A.F., unpublished observations). ‡ the host for these injections, strain PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ (see Methods). This allows simultaneous assay for interference with *gfp* (seen as loss of all fluorescence) and with *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. Double-stranded *gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional body-wall muscles and in the 8 vulval muscles. Lpy-dpy, lumpy-dumpy.

striated muscle cell<sup>3</sup>. Semiquantitative correlations between *unc-22* activity and phenotype of the organism have been described<sup>8</sup>: decreases in *unc-22* activity produce an increasingly severe twitching phenotype, whereas complete loss of function results in the additional appearance of muscle structural defects and impaired motility.

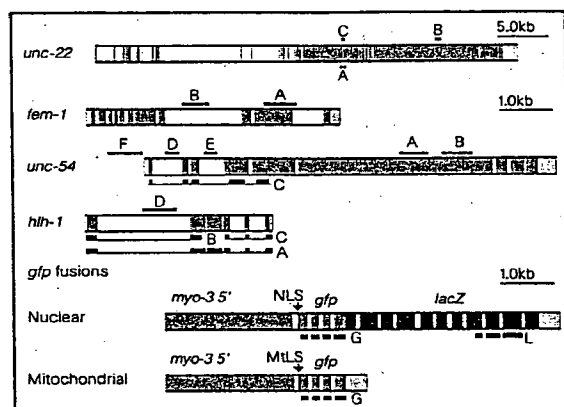
Purified antisense and sense RNAs covering a 742-nucleotide segment of *unc-22* had only marginal interference activity, requiring a very high dose of injected RNA to produce any observable effect (Table 1). In contrast, a sense-antisense mixture produced highly effective interference with endogenous gene activity. The mixture was at least two orders of magnitude more effective than either single strand alone in producing genetic interference. The lowest dose of the sense-antisense mixture that was tested, ~60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. Expression of *unc-22* begins in embryos

containing ~500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

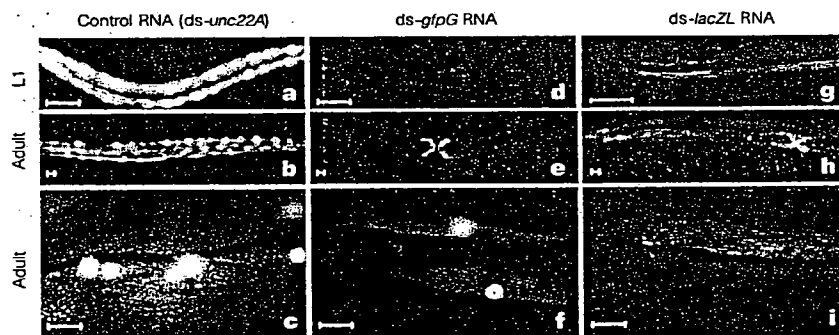
The potent interfering activity of the sense-antisense mixture could reflect the formation of double-stranded RNA (dsRNA) or, conceivably, some other synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double-stranded. The dsRNA was gel-purified from the annealed mixture and found to retain potent interfering activity. Although annealing before injection was compatible with interference, it was not necessary. Mixing of sense and antisense RNAs in low-salt concentrations (under conditions of minimal dsRNA formation) or rapid sequential injection of sense and antisense strands were sufficient to allow complete interference. A long interval (>1 h) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in interfering activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the opposite strand.

A question of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic-response mechanism<sup>10</sup>. Conceivably, our sense-antisense synergy might have reflected a non-specific potentiation of antisense effects by such a panic mechanism. This is not the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of single *unc-22*-RNA strands to mediate inhibition (data not shown). We also investigated whether double-stranded structure could potentiate interference activity when placed in *cis* to a single-stranded segment. No such potentiation was seen: unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate interference. Thus, we have only observed potentiation of interference when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by interference using *unc-22* dsRNA was extremely specific. Progeny of injected animals exhibited behaviour that precisely mimics loss-of-function mutations in *unc-22*. We assessed target specificity of dsRNA effects using three additional genes with well characterized phenotypes (Fig. 1, Table 1). *unc-54* encodes a body-wall-muscle heavy-chain isoform of myosin that is required for full muscle contraction<sup>7,11,12</sup>; *fem-1* encodes an ankyrin-repeat-containing protein that is required in hermaphrodites for sperm production<sup>13,14</sup>; and *hlh-1* encodes a *C. elegans* homologue of myoD-family proteins that is required for proper body shape and motility<sup>15,16</sup>. For each of these genes, injection of related dsRNA produced progeny broods exhibiting



**Figure 1** Genes used to study RNA-mediated genetic interference in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (grey and filled boxes, exons; open boxes, introns; patterned and striped boxes, 5' and 3' untranslated regions. *unc-22*, ref. 9, *unc-54*, ref. 12, *fem-1*, ref. 14, and *hlh-1*, ref. 15). Each segment of a gene tested for RNA interference is designated with the name of the gene followed by a single letter (for example, *unc-22C*). These segments are indicated by bars and upper-case letters above and below each gene. Segments derived from genomic DNA are shown above the gene; segments derived from cDNA are shown below the gene. NLS, nuclear-localization sequence; MtLS, mitochondrial localization sequence.



**Figure 2** Analysis of RNA-interference effects in individual cells. Fluorescence micrographs show progeny of injected animals from GFP-reporter strain PD4251. a-c, Progeny of animals injected with a control RNA (double-stranded (ds)-*unc22A*). a, Young larva, b, adult, c, adult body wall at high magnification. These GFP patterns appear identical to patterns in the parent strain, with prominent fluorescence in nuclei (nuclear-localized GFP-LacZ) and mitochondria (mitochondrially targeted GFP). d-f, Progeny of animals injected with ds-*gfpG*. Only a single active cell is seen in the larva in d, whereas the entire vulval

musculature expresses active GFP in the adult animal in e, f. Two rare GFP-positive cells in an adult: both cells express both nuclear-targeted GFP-LacZ and mitochondrial GFP. g-i, Progeny of animals injected with ds-*lacZL* RNA: mitochondrial-targeted GFP seems unaffected, while the nuclear-targeted GFP-LacZ is absent from almost all cells (for example, see larva in g). h, A typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars represent 20 μm.

the known null-mutant phenotype, whereas the purified single RNA strands produced no significant interference. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from interference with the corresponding gene. The exception (segment *unc54C* which led to an embryonic- and larval-arrest phenotype not seen with *unc-54*-null mutants) was illustrative. This segment covers the highly conserved myosin-motor domain, and might have been expected to interfere with activity of other highly related myosin heavy-chain genes<sup>17</sup>. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments (Table 1; and our unpublished observations) have all been limited to those expected from previously characterized null mutants.

The pronounced phenotypes seen following dsRNA injection indicate that interference effects are occurring in a high fraction of

cells. The phenotypes seen in *unc-54* and *hlh-1* null mutants, in particular, are known to result from many defective muscle cells<sup>11,16</sup>. To examine interference effects of dsRNA at a cellular level, we used a transgenic line expressing two different green fluorescent protein (GFP)-derived fluorescent-reporter proteins in body muscle. Injection of dsRNA directed to *gfp* produced marked decreases in the fraction of fluorescent cells (Fig. 2). Both reporter proteins were absent from the affected cells, whereas the few cells that were fluorescent generally expressed both GFP proteins.

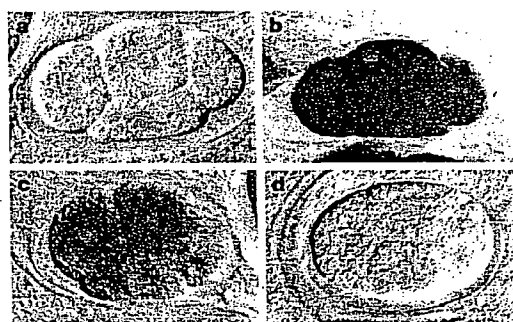
The mosaic pattern observed in the *gfp*-interference experiments was nonrandom. At low doses of dsRNA, we saw frequent interference in the embryonically derived muscle cells that are present when the animal hatches. The interference effect in these differentiated cells persisted throughout larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically derived striated muscles are born during early larval stages and these were more resistant to interference. These cells have come through additional divisions (13–14 divisions versus 8–9 divisions for embryonic muscles<sup>18,19</sup>). At high concentrations of *gfp* dsRNA, we saw interference in virtually all striated body-wall muscles, with occasional lone escaping cells, including cells born during both embryonic and postembryonic development. The non-striated vulval muscles, which are born during late larval development, appeared to be resistant to interference at all tested concentrations of injected dsRNA.

We do not yet know the mechanism of RNA-mediated interference in *C. elegans*. Some observations, however, add to the debate about possible targets and mechanisms.

First, dsRNA segments corresponding to various intron and promoter sequences did not produce detectable interference (Table 1). Although consistent with interference at a post-transcriptional level, these experiments do not rule out interference at the level of the gene.

Second, we found that injection of dsRNA produces a pronounced decrease or elimination of the endogenous mRNA transcript (Fig. 3). For this experiment, we used a target transcript (*mex-3*) that is abundant in the gonad and early embryos<sup>20</sup>, in which straightforward *in situ* hybridization can be performed<sup>5</sup>. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3*. In contrast, animals into which purified *mex-3* antisense RNA was injected retained substantial endogenous mRNA levels (Fig. 3d).

Third, dsRNA-mediated interference showed a surprising ability to cross cellular boundaries. Injection of dsRNA (for *unc-22*, *gfp* or *lacZ*) into the body cavity of the head or tail produced a specific and robust interference with gene expression in the progeny brood (Table 2). Interference was seen in the progeny of both gonad arms, ruling out the occurrence of a transient 'nicking' of the gonad



**Figure 3** Effects of *mex-3* RNA interference on levels of the endogenous mRNA. Interference contrast micrographs show *in situ* hybridization in embryos. The 1,262-nt *mex-3* cDNA clone<sup>20</sup> was divided into two segments, *mex-3A* and *mex-3B*, with a short (325-nt) overlap (similar results were obtained in experiments with no overlap between interfering and probe segments). *mex-3B* antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 h before fixation and *in situ* hybridization (ref. 5; B. Harfe and A.F., unpublished observations). The *mex-3B* dsRNA produced 100% embryonic arrest, whereas >90% of embryos produced after the antisense injections hatched. Antisense probes for the *mex-3A* portion of *mex-3* were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). four-cell-stage embryos are shown; similar results were observed from the one to eight cell stage and in the germ line of injected adults. a, Negative control showing lack of staining in the absence of the hybridization probe. b, Embryo from uninjected parent (showing normal pattern of endogenous *mex-3* RNA<sup>20</sup>). c, Embryo from a parent injected with purified *mex-3B* antisense RNA. These embryos (and the parent animals) retain the *mex-3* mRNA, although levels may be somewhat less than wild type. d, Embryo from a parent injected with dsRNA corresponding to *mex-3B*; no *mex-3* RNA is detected. Each embryo is approximately 50  $\mu$ m in length.

**Table 2** Effect of site of injection on interference in injected animals and their progeny

dsRNA	Site of injection	Injected-animal phenotype	Progeny phenotype
None	Gonad or body cavity	No twitching	No twitching
None	Gonad or body cavity	Strong nuclear and mitochondrial GFP expression	Strong nuclear and mitochondrial GFP expression
<i>unc22B</i>	Gonad	Weak twitchers	Strong twitchers
<i>unc22B</i>	Body-cavity head	Weak twitchers	Strong twitchers
<i>unc22B</i>	Body-cavity tail	Weak twitchers	Strong twitchers
<i>gfpG</i>	Gonad	Lower nuclear and mitochondrial GFP expression	Rare or absent nuclear and mitochondrial GFP expression
<i>gfpG</i>	Body-cavity tail	Lower nuclear and mitochondrial GFP expression	Rare or absent nuclear and mitochondrial GFP expression
<i>lacZL</i>	Gonad	Lower nuclear GFP expression	Rare or absent nuclear-GFP expression
<i>lacZL</i>	Body-cavity tail	Lower nuclear GFP expression	Rare or absent nuclear-GFP expression

The GFP-reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ, was used for injections. The use of this strain allowed simultaneous assay for interference with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence) and *unc-22* (twitching). Body-cavity injections into the tail region were carried out to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior body cavity. An equivalent set of injections was also performed into a single gonad arm. The entire progeny broods showed phenotypes identical to those described in Table 1. This included progeny of both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could be partly due to the persistence of products already present in the injected adult. After injection of double-stranded *unc22B*, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21 out of 21) of these animals. Similar effects (not shown) were seen with double-stranded *unc22A*. Injections of double-stranded *gfpG* or double-stranded *lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of double-stranded *gfpG* and double-stranded *lacZL* produced no twitching, whereas injections of double-stranded *unc22A* produced no change in the GFP-fluorescence pattern.

in these injections. dsRNA injected into the body cavity or gonad of young adults also produced gene-specific interference in somatic tissues of the injected animal (Table 2).

The use of dsRNA injection adds to the tools available for studying gene function in *C. elegans*. In particular, it should now be possible functionally to analyse many interesting coding regions<sup>21</sup> for which no specific function has been defined. Although the effects of dsRNA-mediated interference are potent and specific we have observed several limitations that should be taken into account when designing RNA-interference-based experiments. First, a sequence shared between several closely related genes may interfere with several members of the gene family. Second, it is likely that a low level of expression will resist RNA-mediated interference for some or all genes, and that a small number of cells will likewise escape these effects.

Genetic tools are available for only a few organisms. Double-stranded RNA could conceivably mediate interference more generally in other nematodes, in other invertebrates, and, potentially, in vertebrates. RNA interference might also operate in plants: several studies have suggested that inverted-repeat structures or characteristics of dsRNA viruses are involved in transgene-dependent co-suppression in plants<sup>22,23</sup>.

There are several possible mechanisms for RNA interference in *C. elegans*. A simple antisense model is not likely: annealing between a few injected RNA molecules and excess endogenous transcripts would not be expected to yield observable phenotypes. RNA-targeted processes cannot, however, be ruled out, as they could include a catalytic component. Alternatively, direct RNA-mediated interference at the level of chromatin structure or transcription could be involved. Interactions between RNA and the genome, combined with propagation of changes along chromatin, have been proposed in mammalian X-chromosome inactivation and plant-gene co-suppression<sup>22,24</sup>. If RNA interference in *C. elegans* works by such a mechanism, it would be new in targeting regions of the template that are present in the final mRNA (as we observed no phenotypic interference using intron or promoter sequences). Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose. Genetic interference by dsRNA could be used by the organism for physiological gene silencing. Likewise, the ability of dsRNA to work at a distance from the site of injection, and particularly to move into both germline and muscle cells, suggests that there is an effective RNA-transport mechanism in *C. elegans*. □

## Methods

**RNA synthesis and microinjection.** RNA was synthesized from phagemid clones by using T3 and T7 polymerase<sup>6</sup>. Templates were then removed with two sequential DNase treatments. When sense-, antisense-, and mixed-RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original 'sense' and 'antisense' preparations. Nonetheless, RNA species comprising <10% of purified RNA preparations would not have been observed. Without gel purification, the 'sense' and 'antisense' preparations produced notable interference. This interference activity was reduced or eliminated upon gel purification. In contrast, sense-plus-antisense mixtures of gel-purified and non-gel-purified RNA preparations produced identical effects.

Sense/antisense annealing was carried out in injection buffer (ref. 27) at 37 °C for 10–30 min. Formation of predominantly double-stranded material was confirmed by testing migration on a standard (nondenaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for dsRNA of the appropriate length. Co-incubation of the two strands in a lower-salt buffer (5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of dsRNA *in vitro*. Non-annealed sense-plus-antisense RNAs for *unc22B* and *gfpG* were tested for RNA interference and found to be much more active than the individual single strands, but twofold to fourfold less active than equivalent preannealed preparations.

After preannealing of the single strands for *unc22A*, the single electrophoretic species, corresponding in size to that expected for the dsRNA, was purified using two rounds of gel electrophoresis. This material retained a high degree of interference activity.

Except where noted, injection mixes were constructed so that animals would receive an average of  $0.5 \times 10^6$  to  $1.0 \times 10^6$  RNA molecules. For comparisons of sense, antisense, and double-stranded RNA activity, equal masses of RNA were injected (that is, dsRNA was used at half the molar concentration of the single strands). Numbers of molecules injected per adult are approximate and based on the concentration of RNA in the injected material (estimated from ethidium bromide staining) and the volume of injected material (estimated from visible displacement at the site of injection). It is likely that this volume will vary several-fold between individual animals; this variability would not affect any of the conclusions drawn from this work.

**Analysis of phenotypes.** Interference with endogenous genes was generally assayed in a wild-type genetic background (N2). Features analysed included movement, feeding, hatching, body shape, sexual identity, and fertility. Interference with *gfp* (ref. 25) and *lacZ* activity was assessed using *C. elegans* strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccl4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP); pSAK2 (*myo-3* promoter driving a nuclear-targeted GFP–*LacZ* fusion); and a *dpy-20* subclone<sup>26</sup> as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing easy distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was done as described<sup>27</sup>. Body-cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells is also effective, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16-h intervals. This yields a series of semisynchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short 'clearance' interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. Second, after the clearance period, individuals that show the interference phenotype are produced. Third, after injected animals have produced eggs for several days, gonads can in some cases 'revert' to produce incompletely affected or phenotypically normal progeny.

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Correspondence and requests for materials should be addressed to A.F. (e-mail: [fire@mail1.ciwemb.edu](mailto:fire@mail1.ciwemb.edu)).

## Role of the histone deacetylase complex in acute promyelocytic leukaemia

Richard J. Lin<sup>††</sup>, Laszlo Nagy<sup>††</sup>, Satoshi Inoue<sup>†</sup>, Wenlin Shao<sup>§</sup>, Wilson H. Miller Jr<sup>§</sup> & Ronald M. Evans<sup>††</sup>

<sup>\*</sup> Howard Hughes Medical Institute, and <sup>†</sup> The Salk Institute for Biological Studies, La Jolla, California 92037, USA

<sup>‡</sup> Graduate Program in Molecular Pathology, University of California San Diego, School of Medicine, La Jolla, California 92093, USA

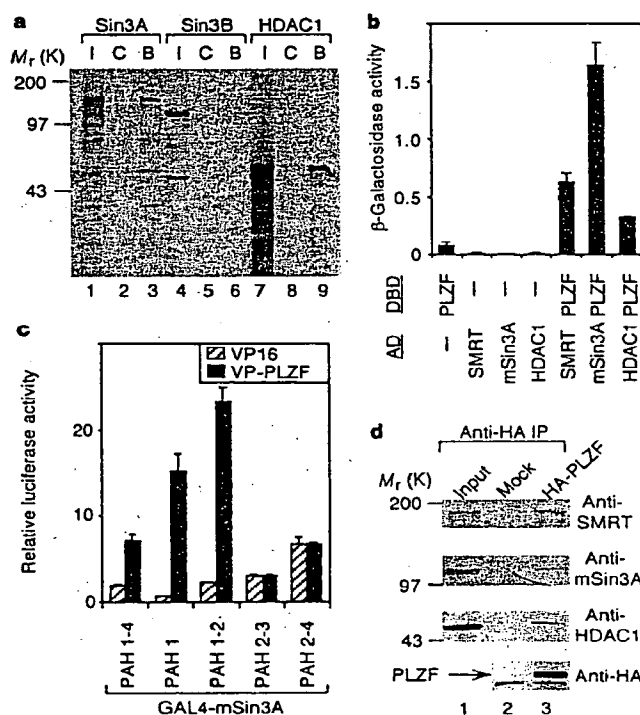
<sup>§</sup> Lady Davis Institute for Medical Research, McGill University Departments of Oncology and Medicine, Montreal, Quebec, Canada H3T 1E2

Non-liganded retinoic acid receptors (RARs) repress transcription of target genes by recruiting the histone deacetylase complex<sup>1–3</sup> through a class of silencing mediators termed SMRT or N-CoR<sup>4,5</sup>. Mutant forms of RAR $\alpha$ , created by chromosomal translocations with either the PML (for promyelocytic leukaemia)<sup>6–8</sup> or the PLZF (for promyelocytic leukaemia zinc finger)<sup>9,10</sup> locus, are oncogenic and result in human acute promyelocytic leukaemia (APL). PML–RAR $\alpha$  APL patients achieve complete remission following treatments with pharmacological doses of retinoic acids (RA); in contrast, PLZF–RAR $\alpha$  patients respond very poorly, if at all<sup>11</sup>. Here we report that the association of these two chimaeric receptors with the histone deacetylase (HDAC) complex helps to determine both the development of APL and the ability of patients to respond to retinoids. Consistent with these observations, inhibitors of histone deacetylase dramatically potentiate retinoid-induced differentiation of RA-sensitive, and restore retinoid responses of RA-resistant, APL cell lines. Our findings suggest that oncogenic RARs mediate leukaemogenesis through aberrant chromatin acetylation, and that pharmacological manipulation of nuclear receptor co-factors may be a useful approach in the treatment of human disease.

Because both PML–RAR $\alpha$  and PLZF–RAR $\alpha$  inhibit normal retinoid signalling<sup>6–14</sup>, we reasoned that identification of factors associated with these proteins might provide mechanistic insights into their oncogenic functions. PLZF–RAR $\alpha$  retains the autonomous repression domain, the BTB/POZ (for bric-à-brac/tramtrack/broad complex, poxvirus and zinc-finger) domain, from PLZF<sup>15</sup>. Because deletion of this domain abolishes the biological functions of PLZF–RAR $\alpha$  *in vivo*<sup>16,17</sup>, we investigated whether it might associate directly with components of the nuclear receptor co-repressor complex<sup>1–3</sup>. By using an *in vitro* interaction assay, we found that radiolabelled full-length mSin3A and histone deacetylase 1 (HDAC1), but not mSin3B, were specifically retained on matrix-

bound fusion proteins of glutathione S-transferase with the BTB/POZ domain of PLZF (GST–PLZF; Fig. 1a). Results from a yeast two-hybrid assay showed that PLZF interacts with all known components of the co-repressor complex *in vivo* (Fig. 1b). We further mapped the PLZF interaction domain in mSin3A to the paired amphipathic helix 1 (PAH1, residues 112–192) by a mammalian two-hybrid assay (Fig. 1c). Finally, using a co-immunoprecipitation assay from nuclear extracts of transfected CV1 cells, we confirmed that PLZF, SMRT, mSin3A and HDAC1 form a complex in mammalian cells (Fig. 1d). These results, together with the finding that SMRT interacts with another BTB/POZ oncoprotein, LAZ3/BCL6 (ref. 18), demonstrated that this family of transcriptional factors recruits histone deacetylases to repress transcription and implicates histone deacetylases in cellular transformation.

By using a yeast two-hybrid assay, we demonstrated that PLZF–RAR $\alpha$  interacts directly with both SMRT and mSin3A, whereas PML–RAR $\alpha$  interacts only with SMRT<sup>19</sup> (Fig. 2B). Most importantly, we showed using a co-immunoprecipitation assay that HDAC1 exists in a complex with either PLZF–RAR $\alpha$  or PML–RAR $\alpha$  in transfected CV1 cells (Fig. 2C). Furthermore, a similar assay using nuclear extracts of the NB4 cells established from a patient with t(15:17) APL<sup>20</sup> indicated that endogenous HDAC1 can be co-precipitated with an anti-PML antibody (Fig. 2D). The presence of endogenous PML–RAR $\alpha$  was confirmed by immunoblotting analyses using anti-PML and anti-RAR $\alpha$  antibodies (data



**Figure 1** Association of co-repressors and HDAC1 with PLZF. **a**, SDS-PAGE analysis of <sup>35</sup>S-labelled mSin3A, mSin3B or HDAC1 proteins retained on immobilized GST–PLZF affinity matrices. I, 20% input; C, GST control; B, bound. **b**, Interactions between PLZF and full-length SMRT, mSin3A or HDAC1 in a yeast two-hybrid assay. **c**, Interactions between GAL4–DBD fusions of different PAHs of mSin3A and VP16 fusion of PLZF are analysed by a mammalian two-hybrid assay in CV1 cells. **d**, PLZF associates with the histone deacetylase complex *in vivo*. CV1 cells were transfected with either vectors only (mock) or plasmids encoding HA–PLZF, SMRT, mSin3A and HDAC1 (HA–PLZF) and nuclear extracts were immunoprecipitated (IP) using anti-HA antibodies followed by immunoblotting analyses using antibodies against SMRT, mSin3A and HDAC1. In lane 1, ~100  $\mu$ g of nuclear extract was applied to ascertain the positions of blotted proteins (input).

## **X. RELATED PROCEEDINGS APPENDIX**

The Appellants' legal representative, or assignee, does not know of any other appeal or interferences which will affect or be directly affected by or have bearing on the Board's decision in the pending appeal. However, for the sake of completeness, Appellants note the following matters which may be of interest to the Board.

A suggestion of interference pursuant to 37 C.F.R. § 41.202 has been filed in related co-pending application 11/364,183. At the time of this appeal, the interference has not been declared.

An appeal has been filed in the re-examination of U.S. Patent No. 6,573,099, which is not directly related to the present application, but is owned by the same real party in interest. The reexamination is being conducted under application numbers 90/007,247 and 90/008,096 (merged). The appeal has been forwarded to the Board of Patent Appeals and Interferences for docketing.

There are no relevant decisions in any related appeal or interference.